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(54) Title: GENETICALLY ENGINEERED VACCINES FOR MYCOBACTERIA

(57) Abstract

Recombinant pox viruses capable of expressing mycobacterial antigens and eliciting protective immune response against mycobacteria. The viruses are useful as live vaccines against mycobacteria.

GENETICALLY ENGINEERED VACCINES
FOR MYCOBACTERIA

Background

The mycobacteria are a group of organisms which
range from innocuous and ubiquitious inhabitants of
water and soil to the highly pathogenic organisms
which are responsible for tuberculosis and leprosy.
These are both chronic infectious diseases of humans
and are characterized by intracellular infection and
slowly evolving granulomatous lesions resulting in
extreme tissue destruction.

M. leprae was the first bacterium shown to be
associated with human disease. It appears to be
extremely adapted to humans; it has not been success-
fully cultivated on artificial media and this
failure makes it an extremely difficult organism to
study and has hindered vaccine development. For-
tunately, it is not highly contagious. M. tuber-
culosis has been cultivated in vitro and is patho-
genic for other animals. Recently, a variety of
other Mycobacterium species have been implicated in
human diseases.

The usual immunologic response to mycobacterial
infections in humans is a delayed-type hyper-
sensitivity (DTH) which is the basis for diagnostic
tests. This DTH response is characteristic of
cell-mediated responses, the main mode of immunity.
Increased resistance to infection in test animals
can be passively transferred with

viable lymphocytes but not with serum. Humoral
immunity plays little if any role in protection and
is currently not of diagnostic importance. Con-
tributing to the ineffectiveness of humoral immunity
05 is the fact that mycobacterial species infect and
multiply within macrophages.

Due to the recent success in cultivating M.
leprae in limited quantities in armadillos, investi-
gators have isolated M. leprae DNA. A variety of M.
10 leprae specific antigens have been identified and
cloned recently. (Young, R. et al., Nature 316: 450
(1985)). Several of these antigens have been shown
to stimulate M. leprae specific T cell clones
(Ottenhoff et al., Nature 319:66 (1986); Mustafa et
15 al., Nature 319:63 (1986)).

Pox viruses of which vaccinia is the prototype
are extremely large, complex DNA viruses which
replicate solely within the cytoplasm of an infected
host cell. Almost two hundred years ago, Edward
20 Jenner proved that cowpox was an effective and safe
vaccine to protect against smallpox. Vaccinia virus
(similar to cowpox) has subsequently been used for
the worldwide eradication of smallpox. Its
effectiveness in the smallpox eradication program
25 was due to relative safety, ease of use, stability,
ease of administration and low cost.

Of all of the viruses which infect animals, pox
viruses have several characteristics which uniquely
suit them for use as eukaryotic expression vectors
30 for generating new recombinant live vaccines. (U.S.

Patent No. 4,603,112: Modified Vaccinia Viruses.
See also, Panicali, et al., _Proc. Natl. Acad. Sci._
USA _80_: 5364 (1983); Smith et al. , _Proc. Natl._
Acad. Sci. USA _80_: 7155 (1983); Cremer, et al.,
05 _Science_ _228_: 737 (1985).

The majority of recombinant pox viruses have
contained genes coding for either viral or nonbac-
terial parasitic antigens.

Disclosure of the Invention

10   This invention pertains to recombinant pox
viruses capable of expressing mycobacterial anti-
gens, particularly mycobacterial antigens capable of
eliciting a protective immune response against
mycobacteria, to methods of producing the recom-
15 binant pox virus, to intermediate DNA vectors which
recombine with pox virus _in vivo_ to produce the
modified pox viruses and to methods of vaccinating a
host with the recombinant pox virus to elicit
protective immunity against mycobacteria in the
20 host. This invention also relates to the uses of
mycobacterial antigens expressed by these recom-
binant vaccinia viruses in cultured cells as vac-
cines or for diagnostic reagents. This invention
also pertains to a method of expressing foreign
25 proteins as the result of gene fusions with
endogenous vaccinia (poxvirus) genes.

Recombinant pox virus capable of expressing
mycobacterial antigens are produced by integrating
into the pox virus genome a gene or genes encoding

the mycobacterial antigen(s) of interest. The
mycobacterial gene is inserted into a region of the
pox virus genome which is nonessential for replica-
tion of the pox virus. The gene is inserted into

05 the pox virus genome in association with a pox virus
promoter to direct its expression.

The mycobacterial gene is integrated into the
pox viral genome by an _in vivo_ recombination event
between an intermediate DNA vector carrying the

10 mycobacterial gene and pox virus. In essence, the
intermediate DNA vector contains the mycobacterial
gene linked to a pox viral promoter located within a
DNA sequence homologous to a region of the pox viral
genome which is nonessential for replication of the

15 pox virus. Thus, the vector comprises:

     a. a prokaryotic origin of replication;

     b. a pox viral promoter;

     c. a gene encoding a mycobacterial antigen
under the direction of the pox viral promoter;

20   d. DNA sequences of the pox virus into which
the gene encoding the mycobacterial antigen is to be
integrated. These DNA sequences flank the promoter
and structural gene at both the 5' and 3' end and
are homologous to the region of the pox virus genome

25 where the mycobacterial gene is to be inserted.

In situations where the mycobacterial gene of
interest contains a translational start signal which
is not recognized as such by eukaryotic cells (e.g.
GTG), the vector can be designed to contain a fused

30 gene construct to allow for expression of the

mycobacterial antigen. This can be accomplished by
linking, in proper reading frame, the mycobacterial
gene containing the translational start signal to a
DNA sequence containing an ATG start signal. For
05 example, the mycobacterial gene can be linked to a
pox gene or portion thereof which contains an ATG
translational start signal recognizable by the
eukaryotic host. This linkage can be made via a DNA
linking sequence. In such embodiments, the fused
10 gene encodes a tripartite fusion protein comprised
of the mycobacterial protein with amino acids
encoded by the pox gene and the DNA linker attached
at its N terminus.

Recombination of the DNA vector and the pox
15 virus is achieved in an appropriate host cell.
Appropriate host cells for in vivo recombination are
eukaryotic cells which are 1) transfectable by the
DNA vector and 2) infectable by pox virus. The host
cell is infected with the pox virus and then is
20 transfected with the DNA vector. Virus is allowed
to replicate in the host cell and recombination
occurs in vivo resulting in insertion of the
mycobacterial gene into the pox virus genome. The
viral progeny is isolated away from the wild type
25 virus. When a selectable marker has been
co-integrated with the mycobacterial antigen,
expression of the selectable marker provides a basis
for selection of recombinant virus containing
integrated mycobacterial DNA. Other methods of
30 selection include detection of integrated

mycobacterial gene by hybridization with homologous
DNA probes or selection for absence of the product
of the viral gene into which the DNA vector has been
inserted.

05   The recombinant virus is a virus which
expresses in tissue culture and in an inoculated
host the mycobacterial antigen of interest. The
virally-expressed antigen will trigger cell-mediated
and humoral immunity against the mycobacterium from
10 which the antigen is derived. Moreover, certain
antigens which are shared antigenic determinants may
provide cross-protection against the various myco-
bacteria.

        In preferred form, the DNA vector for recom-
15 bination with pox virus also contains a gene which
encodes a selectable marker which permits selection
of viral recombinants containing the inserted
mycobacterial DNA. Thus, the vector will contain
these additional elements located between the
20 flanking pox viral sequences:

        e. a second pox viral promoter; and
        f. a gene encoding a selectable marker, the
gene being under the direction of the second pox
viral promoter.

25   There are a number of advantages to creating a
recombinant vaccinia virus expressing mycobacterial
genes to use as a live vaccine. There is a long
history of vaccinia virus being successfully and
safely used in the eradication of smallpox.
30 Vaccination with a live virus would also stimulate

cell-mediated and humoral immunity. The proteins
that are expressed are expected to be appropriately
modified, and if the required signals are present,
they might also be localized to the proper regions
05   of the cell or cellular membrane (Stephens et al.,
EMBO J 5:237 (1986)). In addition, it has been
shown that vaccinia virus has the capability to
recombine in at least 25Kb of DNA (Smith and Moss,
Gene 25:21 (1983)); this amount of DNA can include a
10   number of foreign genes encoding various antigens.
These antigens could all be from the same organism
or from a number of different organisms. Thus, a
single vaccinia virus isolate could serve as a
polyvalent vaccine against one or more pathogens
15   (Perkus et al., _Science_ 229:981 (1985)). Vaccinia
virus vaccines can be manufactured inexpensively and
the virus has been shown to be stable upon storage
under normal field conditions. The vaccine can also
be administered relatively simply. Finally, a
20   recombinant vaccine utilizing vaccinia virus would
avoid the problems associated with vaccinating with
live attenuated or killed bacteria: these strains
may not be properly killed or can become virulent.
It is expected that live viral vaccines would also
25   be more immunogenic than subunit vaccines.

Brief Description of the Figures
        The Figures 1-4 show schematically the con-
struction of the plasmid pAbT6007, a plasmid con-
taining the 65K antigen of _M. leprae_ which

recombines with unmodified vaccinia virus in vivo to
yield a modified vaccinia virus which can express
the 65Kd antigen (in the form of a fusion protein).

Figure 1 shows the construction of plasmid
pAbT6000 which contains the gene encoding the 65
Kilodalton (Kd) antigen of M. leprae.

Figure 2 shows the construction of plasmid
pDP502 which contains the BamF promoter of vaccinia
and the lacZ gene adjacent thereto.

Figure 3 shows the construction of plasmid
pAbT402 which contains, in order, thymidine kinase
(TK) sequences of vaccinia, the vaccinia promoter
for the 11Kd antigen of vaccinia and a portion of
the 11K gene, the BamF-lacZ construct from pDP502
and additional TK sequences.

Figure 4 shows the final steps in construction
of pAbT6007-the insertion of the gene for the 65K
antigen of M. leprae (from pAbT6000) downstream of
the 11K gene portion via a linker in proper frame
with the 11K promoter.

Figure 5 shows the DNA sequence at the junc-
tions of fusion of the 11K gene, the linker DNA and
the gene for the 65K antigen of M. leprae and shows
the predicted amino acid sequence at the junctions.

Figure 6 shows the construction of plasmids
pAbT8000 and pAbT8005, precursors for construction
of pAbT8008.

Figure 7 shows the construction of plasmid
pAbT8008 which contains the vaccinia 7.5K promoter
driving expression of the gene for the tuberculosis

65Kd protein and the Bam F promoter driving
expression of the lacZ gene.

Figure 8 shows the construction of plasmid
pAbT8032 which contains the gene for the leprosy
05   65Kd antigen driven by the vaccinia 7.5K promoter.

Figure 9 shows the construction of plasmid
pAbT8501 which contains the gene for the leprosy
12Kd antigen under control of the 7.5K promoter of
vaccinia.

10   Detailed Description of the Invention

1.   Genes encoding mycobacterial antigens

Genes encoding mycobacterial antigens can be
obtained from the genomic DNA of a mycobacterium or
from available clones containing the genes.   As
15   mentioned, the isolation and cloning of M. leprae
DNA has been described.   See Young, et al., Nature
316:450 (1985), the teachings of which are
incorporated by reference herein.   Procedures for
obtaining the DNA from other mycobacteria, e.g. M.
20   tuberculosis and M. bovis, are reported by
Clarke-Curtiss et al., J. Bact. 161:1093 (1985).
Genes for tuberculosis antigens have aslo been
cloned and analyzed (Young et al. Proc. Natl. Acad.
Sci. USA 82:2583 (1985); Husson and Young, Proc.
25   Natl. Acad. Sci. USA 84:1679 (1987); and Shinnick,
J. Bact. 169:1080 (1987)).

Because cell-mediated responses appears to be
the primary immunological responses involved in

protection against mycobacterial infection, pre-
ferred mycobacterial antigens are those which elicit
a cell-mediated response in addition to a humoral
(antibody) response. The 65Kd, 12Kd, 36Kd and 18Kd
05 antigens of M. leprae, the 65Kd and 19Kd antigen of
M. tuberculosis and the 65Kd of M bovis appear to be
involved in the cell-mediated response to these
mycobacteria. See e.g. Ottenhoff et al., Nature
319:66 (1986); Mustafa et al. Nature 319:63 (1986);
10 Emmrich et al. Abstract, International Cong. of
Immunol. (1986); Oftung et al., J. Immunol. 138:927
(1987); Emmrich et al., J. Exp. Med. 163:1024
(1986). In addition, mycobacterial antigens can be
modified to enhance presentation of the antigen on
15 the surface of virally infected cells. This can be
accomplished by genetically engineering signal and
anchor polypeptide sequences on the antigen.
Specific mycobacterial epitopes (Mehra et al., Proc.
Natl. Acad. Sci. USA 83:7013 (1986); Lamb et al.,
20 EMBO J. 6:1245 (1987)) can also be expressed on the
cell surface with the appropriate signal and anchor
sequences.

Especially preferred antigens are those anti-
gens which are crossreactive between mycobacteria.
25 For example, the 65Kd antigens of M. leprae, M.
tuberculosis, and M. bovis have a significant degree
of homology (Husson and Young, Proc. Natl. Acad.
Sci. USA 84:1679 (1987)). Antigens of this type may
provide cross-protective immunity against various
30 mycobacteria.

2. Pox viruses

A suitable pox virus for generating recombinant
virus includes any members of the pox family which
do not cause significant disease in normal humans or
05 animals. The preferred pox virus is vaccinia virus,
a relatively benign virus which has been used for
years as a vaccine against small pox. General
techniques for integration of heterologous DNA into
vaccinia virus to provide modified vaccinia virus
10 capable of expressing foreign protein encoded by the
heterologous integrated DNA are described by
Paoletti et al. U.S. Patent No. 4,603,112, the
teachings of which are incorporated by reference
herein.

15 3. Preparation of intermediate DNA vectors for
recombination with pox virus

According to the method of this invention,
cloned mycobacterial genes which code for antigenic
proteins are inserted into the genome of a pox virus
20 in such a manner as to allow them to be expressed by
the pox virus along with the expression of the
normal complement of pox virus proteins. This is
accomplished by first constructing a prokaryotic
insertion vector which contains (i) a prokaryotic
25 origin of replication, so that the vector may be
amplified in a prokaryotic host, (ii) DNA sequences
homologous to the region of the pox virus genome
where the mycobacterial gene is to be inserted,
(iii) the mycobacterial gene inserted at a site

within this pox virus sequence and (iv) a pox virus
regulatory sequence (promoter) adjacent to the 5'
end of the mycobacterial gene, constructed in a
manner to allow for transcription of the mycobac-
05   terial gene and subsequent expression. This exact
construction may contain only the vaccinia promoter
sequence or the promoter and adjacent regions of the
pox virus gene from which the promoter was derived.
The viral promoter and virally-derived flanking
10   sequences are obtained from genomic DNA or cloned
fragments. The mycobacterial gene is obtained as
described above. The vector backbone can be derived
from any of several plasmid vectors capable of
replication in a prokaryotic host, e.g., pBR322
15   (Sutcliffe, Cold Spring Harbor Symp, Quant. Biol.
43:77 (1979)), pUC8 (Vieira and Messing, Gené 19:259
(1982) or pEMBL (Dente et al., Nucleic Acids Res.
11:1645 (1982)). The ability to replicate in a
prokaryotic host provides a means for amplification
20   of the vector to produce sufficient quantities for
transfection of a eukaryotic cell for
recombinations.

In addition, the vector preferably contains a
gene which encodes a marker which will allow selec-
25   tion of recombinant pox viruses containing inte-
grated mycobacterial DNA. The gene encoding the
marker is placed under control of a pox virus
promoter. Several types of marker genes can be
used. A preferred marker gene is the lacZ gene
30   which encodes the enzyme beta-galactosidase. Re-

combinant pox virus will express beta-galactosidase
along with the mycobacterial antigen and beta-galac-
tosidase production is detected as an indication of
insertion and expression of the foreign DNA.
05 Recombinant viruses which express beta-galactosidase
will form blue plaques while wild-type virus not
expressing beta-galactosidase will form clear
plaques. Recombinant viruses which have sequences
inserted into their TK gene will grow in the
10 presence of BUdR while virus containing a wild-type
uninterupted TK gene will be unable to do so (Dubbs
and Kit, _Virol._ __22__:214 (1964); Smith et al., _Proc._
Natl. Acad. Sci. USA __80__:7155 (1983)). Other
selectable markers include genes which confer
15 antibiotic resistance in an infected host cell,
e.g., the Neo^R gene. Infected cells are grown in
media containing the antibiotic at a concentration
toxic for antibiotic sensitive cells. Under these
conditions, cells infected with a recombinant virus
20 expressing the resistance marker will produce virus.
Cells infected with virus not containing the marker
will not produce virus.

    A preferred DNA vector for recombination with
the preferred pox virus vaccinia virus comprises:
25   a.   a vaccinia promoter (e.g., the vaccinia
            11K, 7.5K, 30K, 40K or BamF promoter or
            modified versions of these promoters)
            linked to;
    b.   a structural gene encoding a mycobacterial
30   antigen of interest (e.g., the 65Kd

antigen of <u>M</u>. <u>leprae</u> or <u>M</u>. <u>tuberculosis</u>)
under control of the promoter;

   c.   a second vaccinia promoter (e.g., the BamF
promoter of vaccinia virus) linked to;

05   d.   a gene encoding a selectable marker (e.g.,
the lacZ gene); and

   e.   DNA sequences homologous with a region of
vaccinia nonessential for replication
flanking the construct of elements a-d

10   (e.g., vaccinia thymidine kinase se-
quence).

Some prokaryotic genes contain the transla-
tional initiation codon GTG which is not functional
in eukaryotic systems. This is the case, for

15 example, for the gene encoding the 65Kd antigen of
<u>M</u>. <u>leprae</u>. In some cases, the cloned gene may not
have an initiation codon because it is incomplete
and therefore one must be supplied. In such
instances, the intermediate vector can be designed

20 so that the prokaryotic gene is linked to an ATG
codon. For example, this can be done via a sequence
containing an ATG. Preferably, this is accomplished
by constructing a fused gene containing a promoter
of pox virus and the adjacent coding regions of the

25 pox virus gene from which the promoter is derived.
The promoter and ATG initiation codon can be linked
to the prokaryotic gene by a DNA linker. In this
case, the fused gene construct would encode a
tripartite fusion protein and the prokaryotic

30 antigen would be expressed as such. The tripartite

gene product would comprise (from N-terminus to
C-terminus):

    a.   amino acids encoded by vaccinia ATG-
         supplying DNA segment;

05   b.   linker encoded amino acids; and

    c.   prokaryotic protein.

The fused gene can be designed to minimize the
number of amino acids of nonmycobacterial origin so
as not to alter significantly immunogenic or other

10 properties of the mycobacterial antigen. Alter-
natively, if it is inconvenient to use the ATG
initiation codon which was originally associated
with the promoter of interest, one can choose a
linker sequence which will provide an in-frame ATG.

15 In such a case, the recombinant protein would be
comprised of amino acids encoded by the linker DNA
and the mycobacterial gene.

As described in more detail in the examples
below, the gene for the 65Kd antigen of M. leprae

20 was inserted into an intermediate vector containing
a) the vaccinia thymidine kinase gene, as the
sequence for homologous recombination, b) the
promoter and codons specifying the first two amino
acids of the 11K gene of vaccinia virus, to direct

25 expression of the 65Kd antigen, and c) the E. coli
B-galactosidase gene under the control of the
vaccinia Bam F promoter, to facilitate identifica-
tion of the recombinant virus by use of a chomogenic
assay. Using this intermediate vector, a

30 recombinant vaccinia virus was created which
expresses the 65Kd antigen. The vaccinia expressed

65Kd antigen was recognized by two monoclonal
antibodies which recognized specific epitopes on the
65Kd antigen.

4. In vivo recombination
05   The intermediate DNA vectors containing the
mycobacterial gene (and the marker gene) flanked by
appropriate pox viral sequences will undergo recom-
bination with pox virus genomic DNA which results in
integration of the flanked gene into the viral
10 genome. Recombination will occur in a eukaryotic
host cell: Appropriate host cells for recombination
are those which are 1) infectable by pox virus and
2) transfectable by the DNA vector. Examples of
such cells are chick embryo fibroblast, CV-1 (monkey
15 kidney cells), HuTK 143 cells (human cells), BSC40
(monkey kidney cells).
        Viral infection is accomplished by standard
techniques for infection of eukaryotic cells with
pox virus.
20   The cells can be transfected with the inter-
mediate vector by any of the conventional techniques
of transfection. These include the technique of
calcium phosphate precipitation, DEAE dextran,
electroporation and protoplast fusion. The pre-
25 ferred technique is the calcium phosphate precipita-
tion technique.
        After infection and subsequent transfection,
the cells are incubated under standard conditions
and virus is allowed to replicate, during which time
30 in vivo recombination occurs between the homologous

pox virus sequences in the intermediate vector and
the pox virus sequences in the genome.

Recombinant viral progeny are then selected by
any of several techniques. The presence of inte-
05 grated mycobacterial DNA can be detected by hybridi-
zation with a labeled DNA probe specific for the
mycobacterial DNA. Alternatively, virus harboring
the mycobacterial gene can be selected on the basis
of inactivation of the viral gene into which foreign
10 DNA was inserted. For example, if the DNA vector is
designed for insertion into the TK gene, viruses
containing integrated DNA will be TK⁻ and can be
selected on this basis. Preferred techniques for
selection, however, are based upon co-integration of
15 a gene encoding a marker or indicator gene as
described above. The preferred indicator gene is
the LacZ gene. Selection of recombinant viruses
expressing B-galactosidase can be done by employing
a chromogenic substrate for the enzyme.

20 5. Vaccine compositions

Recombinant pox viruses expressing mycobac-
terial antigens can be used as live viral vaccines
to vaccinate hosts (humans or animals) susceptible
to mycobacterial disease. Recombinant pox viruses
25 expressing cross-protective antigens such as the
65Kd antigen of M. leprae, M. tuberculosis or M.
bovis may provide cross protective immunity in both
humans or animals. In other instances, it may be
necessary to use recombinant viruses expressing one

or more type specific mycobacterial antigens to
induce sufficient immunogenic response in the
vaccinated host to be protective.

05   Recombinant pox viruses may be produced in
cultured eukaryotic cells. Other techniques may be
used to produce pox virus vaccines e.g. production
of vaccinia virus in calves.

6.   Diagnostic Applications

      The mycobacterial antigens produced during the
10   infection with recombinant pox virus are also of
diagnostic value. For example, during recombinant
pox infection, the M. leprae 65Kd antigen can be
produced; this antigen reacts with monoclonal
antibodies to M. leprae 65Kd antigen to an extent
15   which can be detected by ELISA. This result shows
the feasibility of using M. leprae antigens produced
in pox infected cells for testing for the presence
of M. leprae-specific antibodies and T cells in
humans. This can determine whether or not
20   individuals have been exposed to M. leprae.

      The invention is illustrated further by the
following Examples.

EXAMPLES

MATERIAL & METHODS

E. coli Strains:

        E. coli strains JM101 (Messing et al., Nucl.

05 Acids Res. 9:309 (1981)), MC1060 (Casadaban & Cohen,

    J. Mol. Biol. 138, 179 (1980)) and RR1 (Bolivar et

    al., Gene 2:95 (1977); Peacock et al., Biochem.

    Biophys. Acta 655:243 (1981) were used.

Restriction Enzyme Digestion:

10   Enzymes were obtained from New England Biolabs

    or Boehringer-Mannheim. Digests were performed as

    described (Maniatis, T., Fritsch, E.F. and Sambrook,

    J., 1982, Molecular Cloning; A Laboratory Manual.

    Cold Spring Harbor Laboratory, Cold Spring Harbor,

15 N.Y., pp. 104-105). Digests were incubated at 37°C

    for 1 hr unless otherwise specified.

Treatment of DNA with Calf Intestinal Phosphatase:

        DNA was dephosphorylated in 50mM Tris-HCl,

    Tris-HCl, pH9.0, 1.0mM $MgCl_2$, 0.1mM $ZnCl_2$ and 1mM

20 spermidine with 1ul of calf intestinal phosphatase

    (CIP) (Boehringer Mannheim, 23 units/ul) at 37°C for

    30 min, sometimes followed by a second 30 min incu-

    bation with another 1ul of enzyme.

Treatment of DNA with DNA Polymerase, Large Fragment

25 (Klenow):

        Klenow enzyme was obtained from New England

    Biolabs and used as described (Maniatis, T.,

Fritsch, E.F. and Sambrook, J., 1982. Molecular
Cloning: A Laboratory Manual. Cold Spring Harbor
Laboratory, Cold Spring Harbor, N.Y. pp. 112-113).

Gel-Purification of Restriction Fragments:

05   DNA was fractionated by size on low-melt
agarose (ranging from 0.7% to 3%) gels run in 40mM
Tris-acetate pH8.0, 2mM EDTA. The DNA fragment of
interest was excised from the gel, liquified at 70°C
and diluted in 200mM NaCl, 50mM Tris, pH7.5, 1mM
10   EDTA. The DNA was extracted with phenol, then
phenol:chloroform (1:1) and precipitated with
ethanol.

Isolation of DNA from Agarose Gels using Glass
Powder:

15   The DNA fragment of interest was excised from
an agarose gel. The slice was placed in an
Eppendorf tube, weighed and dissolved at 60°C in 2-3
ml of NaI solution (6.06M NaI which had been
saturated with 1.5g of Na_2SO_3 and then filtered) per
20   gm of gel. After the agarose was dissolved, 2-10 ul
of glass slurry (Vogelstein and Gillespie, Proc.
Natl. Acad. Sci. USA 76:615 (1979)) was added and
allowed to bind to the DNA for 30-60 min on ice.
The following steps were then performed at 4°C:
25   1.   The centrifuge tube was spun for 15 sec to
pellet glass, and the supernatant was removed.
       2.   The glass pellet was resuspended in 1 ml
of NaI solution.

3.   The tube was again centrifuged for 15 sec and the resulting supernatant was discarded.

4.   The pellet was resuspended in 1 ml of ethanol wash solution (50% ethanol, 0.1M NaCl, 10 mM Tris-HCl pH 7.5) which had been stored at -20°C.

5.   The glass suspension was centrifuged and then supernatant was removed.

6.   Steps 4 and 5 were repeated.

Next the pellet was resuspended in 50-100 ul of TE and the DNA was eluted at 37° for 30-60 min. The suspension was centrifuged and the supernatant containing DNA was removed to a new tube. The pellet was resuspended in another 50-100 ul of TE and spun down and this second supernatant was combined with the first one. The DNA was then extracted once with phenol/chloroform, twice with chloroform, and then precipitated with ethanol. The DNA was resuspended in TE.

Ligation of DNA Fragments:

T4 DNA Ligase was obtained from Boehringer Mannheim. Ligations were performed in 50mM Tris-HCl, pH7.4, 10mM $MgCl_2$, 10mM dithiothreitol, 1mM spermidine, 1mM adenosine 5'-trisphosphate, 0.1mg/ml bovine serum albumin, 1u T4 DNA ligase, at 15°C for 30 min to 3 days unless otherwise specified.

Phosphorylation of Linkers and Ligation to DNA:

Linkers and T4 polynucleotide kinase were obtained from New England BioLabs. Linkers were phosphorylated and ligated as described (Maniatis,.
05 T., Fritsch, E.F. and Sambrook, J., 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., pp 396-397).

E. coli Transformation:

E. coli cells were made competent and trans-
10 formed with DNA as described (Maniatis, T., Fritsch, E.F. and Sambrook, J., 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, N.Y., pp 250-251).

Isolation and Purification of Plasmid DNA:

15 .   Preparation of plasmid DNA and purification by cesium chloride-ethidium bromide gradient centrifugation were performed as described (Maniatis, T., Fritsch, E.F. and Sambrook, J., 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor
20 Laboratory, Cold Spring Harbor, N.Y. pp. 90, 91, 93, 94).

Virus and Cells:

CV-1 cells, which are kidney cells derived from the African Green Monkey, were obtained from the
25 American Type Culture Collection (ATCC #CCL70) and grown in Minimal Essential Media (MEM; Gibco) supplemented with 10% fetal calf serum (FCS; Hyclone

Laboratories, Inc.). BSC40 cells were obtained from
B. Roberts (Harvard Medical School, Boston, MA) and
were grown in DME and 10% FSC.

05 Vaccinia virus strain NYCBH (New York City
Board of Health) was obtained from the ATCC (catalog
#ATCC VR-325). The virus stock received from ATCC
was grown on CV-1 cells in MEM-2% FCS, and this
virus was further amplified in spinner cultures of
HeLa-S3 cells (ATCC #CCL2.2) for purification on

10 sucrose gradients and infection in in vivo recom-
bination (IVR) experiments.

Infection and Transfection:

CV-1 cells were plated 24 hr before an IVR at
10^6 cells per 6cm plate. These cells were infected

15 with vaccinia virus at a multiplicity of infection
(MOI) of 0.05 to 2 in a total volume of 200ul of
MEM-2% FCS. Virus was adsorbed for 40 min at 37°C;
during this time the plates were rocked every 10-15
min to distribute the virus and to keep the plates

20 from drying out. After virus adsorption, 3.3ml of
MEM-2% FCS was added to the cells.

Solution A (250mM $CaCl_2$, 25mM HEPES, pH7.12)
was added to 20ug of CsCl gradient-purified DNA
(total volume of 30ul or less) for a final total

25 volume of 250ul. While air was then bubbled through
this solution, 250ul of solution B (280mM NaCl,
1.5mM Na_2HPO_4, 25mM HEPES, pH7.12) was added drop-
wise, and the solution was incubated at room
temperature for 40 min. This solution was then

added dropwise to infected cells while the plate was
gently swirled.

The infected cells which had been transfected
with DNA right after completion of virus adsorption
05 were incubated at 37°C until all the cells were
observed to be infected; typically, cytopathic
effects were seen by 16-20 hr. Virus from the IVR
was harvested by freezing and thawing the plates
three times. Cells were scraped off the plate into
10 the medium, cell debris was removed by centrifu-
gation in a clinical centrifuge for 5 min, and the
supernatant containing the virus was sonicated for
40 sec or vortexed and stored at -80°C.

Plaque Purification of Recombinant Virus:
15   The progeny virus was then titered on a mono-
layer of CV-1 cells on a 6cm plate. Because the DNA
used in the transfection step contained the gene for
lacZ under the regulation of a vaccinia promoter as
well as a gene of interest, i.e. gene encoding
20 antigen, one could detect recombinant virus as blue
plaques in the presence of Bluo-Gal (Bethesda
Research Laboratories). Because in vivo recom-
bination occurs at a low frequency and blue plaques
could be isolated from no more than 250-300 back-
25 ground clear plaques, the virus titer from each IVR
was determined by a titration curve in which the
virus yield was diluted 10^{-1} through 10^{-4} in MEM-2%
FCS.

A 6cm plate of confluent CV-1 cells (passaged
24 hr earlier) was infected with 0.5ml of diluted
virus which was adsorbed for 30 min at 37°C. The
medium was then removed by aspiration and the cells
05   were overlaid with 3ml of Dulbecco's Modified
Eagle's Medium (DME) minus Phenol Red (Gibco) - 0.6%
agarose (Bio-Rad) - 10% FCS - 0.1ug/ml fungizone.
This overlay was made by mixing equal volumes of 2x
(DME minus Phenol Red-FCS-fungizone) with 1.2%
10   agarose. On day 3, another 3ml of overlay
containing the previous mixture and 400ug/ml
Bluo-Gal (from a freshly prepared stock at 20mg/ml
in dimethyl sulfoxide (DMSO)) was placed over the
first agarose overlay. The plates were then
15   screened daily for the number of blue and white
plaques; blue plaques first appeared anywhere from
4-48 hr.
     Having calculated the plaque forming units/ml
(PFU) for the progeny virus in the IVR, one can now
20   set up an infection of 15-20 6cm plates of CV-1
cells and use that amount of virus calculated to
result in 250-300 plaques per plate. This infec-
tion, covering of cells with first and second
overlay and screening of the plaques were all done
25   as described above. Recombinant blue plaques were
picked and placed into 2.0ml MEM-2% FCS and soni-
cated for 40 sec or vortexed several times. A next
round of plaque purification was done by infecting
cells with 20ul to 200ul of this sonicated virus,

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and this was followed by a third and fourth round of
plaque purification.

Virus Amplification:

        After plaque purification, the resulting virus
05 was amplified on a 6cm plate of CV-1 cells. This
    virus was harvested in a total volume of 5ml and the
    cells lysed by 3 cycles of freeze-thawing. Cellular
    debris was removed by centrifugation and the re-
    sulting virus was again amplified first on another
10 6cm plate of CV-1 cells, and then again on a 15cm
    plate of CV-1 cells. The virus particles (in 20ml)
    were then concentrated by layering over a 15ml
    cushion of 36% (w/v) sucrose in 1mM Tris-HCl, pH9.0,
    and centrifuging in the SW28 rotor at 20,000 rpm at
15 4°C for 60 min. The virus pellet was resuspended in
    a small volume of 1mM Tris-HCl, pH9.0. The concen-
    tration of PFU was determined as previously de-
    scribed; in this case dilutions ranged from 10^{-3} to
    10^{-8}.

20   The virus resulting from the infection of the
    15cm plate of CV-1 cells was then used to infect
    approximately 1 liter of HeLa-S3 cells in a spinner
    at an MOI of 1. Virus was trypsinized with an equal
    volume of 0.25% trypsin (Gibco) for 30 min at 37° C
25 in a shaker. HeLa-S3 cells were spun down, resu-
    spended in 20ml Dulbecco's phosphate-buffered saline
    (DPBS: 137mM NaCl, 2.7mM KCl, 1.5mM KH_2PO_4, 8.1mM
    Na_2HPO_4, 4.9mM $MgCl_2$, 9.1mM $CaCl_2$, pH7.4) and
    counted; the cells were then further concentrated by

centrifugation and resuspended in a small volume
(approximately 2ml) of DPBS-1% FCS. The appropriate
amount of trypsinized virus was added to the cells
and adsorbed for 45 min in a shaker at 37°C. The
05 infected cells were then diluted with fresh DME-10%
FCS to a final concentration of approximately 4 x
10^5 cells/ml. After a 3 day incubation at 37°C, the
cells were pelleted, resuspended in approximately
100ml of 1mM Tris-HCl, pH 9.0, and left on ice for
10 30 min. The cells were then homogenized in 25ml
aliquots with 10 strokes each in a Dounce
homogenizer. The cells were pelleted at 6,000 rpm
in the Sorvall SA-600 rotor for 5 min and the
supernatant containing the virus was set aside.
15 The pelleted cells were resuspended in 20 ml of 1mM
Tris-HCl, pH9.0, and homogenized again with five
·strokes of a Dounce homogenizer. The cells were
pelleted at 8,000 rpm in the Sorvall SA-600 rotor
and the supernatant containing the virus was pooled
20 with the first supernatant. At this point, the
virus was frozen at -80°C until further purification
through a sucrose cushion and a sucrose gradient.
Virus was purified by pelleting through a 15 ml
cushion of 36% (w/v) sucrose in 1mM Tris-HCl, pH
25 9.0, at 20,000 rpm at 4°C for 60 min in the SW28
rotor. The virus pellet was resuspended in a small
volume of 1mM Tris-HCl, pH 9.0, and banded on a
25-40% sucrose (w/v) gradient in 1mM Tris-HCl, pH
9.0, in a SW28 rotor at 15,000 rpm at 4°C for 40
30 min. The virus band was collected and repelleted at

20,000 rpm in the SW28 rotor for 60 min at 4°C.   The
pellet was resuspended in 1mM Tris-HCl, pH 9.0 and
subsequently titered.

RNA Dot Blots:

05   Cells were infected at an MOI of 2 and grown
for 16-24 hr.   The medium was removed, cells were
washed twice with PBS and 600 ul lysis buffer (equal
volumes of DMSO mixed with an equal volume of 4 M
guanidinium thiocyanate, 25mM sodium citrate (pH7),

10   0.5%   sarkosyl, 0.1M B-mercaptoethanol was added.
The cells were incubated at 37°C for 30 min and then
scraped and collected into Eppendorf tubes.   The
samples were stored at -20°C.
      The samples were filtered through

15   nitrocellulose on a dot blot manifold (Schleicher
and Schuell) and washed twice with of 200ul 20XSSC
(3M NaCl, 0.3M Na citrate) and once with 2XSSC.   The
filter was air-dried, and baked at 80°C in a vaccum
oven.

20   The filter was prehybridized for 2 hr and
hybridized overnight in 50% formamide, 3XSSC, 5X
Denhardts (1 X Denhardts is 1% Ficoll, 1%
polyvinylpyrrolidone, 1% Bovine Serum Albumin), 0.1%
SDS and 100ug/ml tRNA at 42°C.   The ^{32}p-labeled

25   probe added to the hybridization mix was generated
by oligolabeling (OLB; Feinberg and Vogelstein,
Anal. Biochem. 137:266 (1984); Feinberg and
Vogelstein, Anal. Biochem. 132:6 (1983)) with the
Pharmacia Oligolabeling Kit (#27-9250).

Following hybridization, the filter was washed
twice for 30 min in 3XSSC at room temperature and
then twice for 30 min in 0.1X SSC at 65°C. After
air-drying, the filter was exposed to film for 2
05   days with a screen at -70°C.

RNA Preparation for Hybrid Selections:

CV-1 cells were infected with vaccinia virus
and harvested at various times during infection.
The cells were washed twice in cold PBS which was
10   then removed. The cells were then lysed with
ice-cold 4 M guanidine thiocyanate, 25 mM sodium
citrate (pH 7), 0.5% Sarkosyl, and 0.1M
beta-mercaptoethanol. The cell lysate was then
passed at least four times through a syringe needle
15   to shear DNA, layered onto a 3-ml cushion of 5.7
CsCl-0.1 M EDTA treated with diethylpyrocarbonate,
and centrifuged for 18 h at 15°C in an SW50.1 rotor
at 35,000 rpm. The RNA pellet was washed with 70%
ethanol, suspended in water, and precipitated with
20   ethanol after the addition of 0.1 volume of 2 M
sodium acetate.

Hybrid Selection of RNA:

One ug of linearized denatured plasmid DNA was
spotted onto nitrocellulose filters which were then
25   baked in a vaccum oven at 80°C for 2 hr. The
filters were then incubated with 40 ug RNA in 65%
formamide, 0.01 M Pipes, pH 6.4, 0.4 M NaCl for 3 hr
at 48°. Unhybridized RNA was removed by ten washes

of 1X SSC and 0.5% SDS at 57°C followed by three
washes with 2.5 mM EDTA. Hybridized RNA was
released by boiling the filter for 60 sec in 1 mM
EDTA. This RNA was recovered, precipitated with 10
05 ug tRNA and trnslated in a rabbit reiculocyte
cell-free translation system.

Cell-free Translation of RNA:
        Rabbit reticulocyte lysate was obtained from
Promega Biotec and translation reactions with ^{35}S
10 methionine were done according to their directions.
The translation products were analyzed on SDS
polyacrylamide gels (Laemmli, Nature 227: 680
(1970)) and processed for fluorography using
Enchance (New England Nuclear).

15 Black Plaque Assay: -
        CV-1 cells were plated out one day prior to
infection at $7x10^5$ cells per 6cm dish, in MEM
containing antibiotics and antimycotics (Sigma) and
10% FCS. Twenty-four hours later the cells became
20 confluent ($1x10^6$ cells per dish). Growth medium was
removed from the cells and replaced with 0.5ml of
medium containing the recombinant vaccinia virus
(approximately 100 PFU/plate) or control virus
(NYCBH). Virus was adsorbed to the cells for 30
25 minutes at 37°C, and at the end of this time, the
medium was removed by aspiration and the cells were
overlaid with 0.6% agarose in DME containing 10%FCS

and 0.1ug/ml fungizone. The cells were then
incubated for two days to allow plaques to form.

After the two day incubation, the agarose
overlay was removed, leaving the cell monolayer
05   intact on the dish. The cells were washed three
times with 3ml of DPBS, and were fixed for 15 min at
room temperature, using 3% formaldehyde
(Mallinckrodt) in phosphate-buffered saline (PBS:
137mM NaCl, 2.7mM KCl, 1.5 mM KH_2PO_4, 8.1mM
10   Na_2HPO_4). The monolayer was again washed three
times with 3ml of DPBS, and was then incubated with
a primary antibody, e.g. IIC8 (Gillis and Buchanen,
Infect. Immunity 37, 172 (1982)) or Y1-2 (Engers et
al., Infect. Immunity 48:603 (1985)). These two
15   monoclonal antibodies both received as ascites
fluid, were obtained from Dr. Richard Young, White-
head Institute for Biomedical Research, Cambridge,
Ma). The monoclonal IIC8 was diluted 1:1,270 in 50%
normal goat serum (NGS; Colorado Serum Company) in
20   PBS, and 1ml of this solution was added to the
monolayer, and incubated overnight at 4°C with
constant shaking. When monoclonal antibody Y1-2 was
used, it was diluted 1:500 in 50% NGS, and 1ml of
this solution was incubated with the monolayer for 1
25   hr at 37°C. After the incubation, the antibody
solution was removed by aspiration and the monolayer
was washed three times with 3ml of washing buffer
(20mM Tris-HCl, pH 7.5, 1M NaCl, 0.05% Tween-20).
The cells were then incubated with the secondary
30   antibody, alkaline phosphatase-conjugated

goat anti-mouse IgG (heavy and light chain specific;
Kirkegaard & Perry Laboratories). This antibody was
diluted 1:200 in 10% NGS, and 1ml was incubated with
the monolayer for 1 hr at 37°C. The solution was
05   then removed by aspiration, and the cells were
washed three times with 3ml of washing buffer, and
once with Tris-buffered saline (TBS; 20mM Tris-HCl,
pH7.5, 0.15 M NaCl). Color was developed on plaques
which were expressing the antigen of interest by
10   incubating with a precipitating substrate system,
BCIP/NBT (5-bromo-4-chloro-3indolyl-phosphate and
nitroblue tetrazolium (Kirkegaard & Perry Labora-
tories)). The reagent was made up as per manu-
facturer's instructions, and 2ml was incubated with
15   the monolayer at room temperature until color
developed on the plaques (approximately 30 min).

Live Black Plaque Assay on BSC40 Cells:
        Dishes containing 6 wells were coated with 0.1%
gelatin in PBS. The plates remained at room
20   temperature for two hr after which the gelatin was
removed and the plates were air-dried in the hood.
        BSC40 cells (5 X 10^5/3.5cm well) were plated
and the following day the cells were infected with
approximately 100 PFU of virus. The virus was
25   adsorbed for 30 min at 37°C and then removed. Four
ml of DME - 2% FCS (heat inactivated) were added.
Two days later, the live black plaque assay was
done.

The live black plaque assay was done under
sterile conditions. The medium was removed from the
cells and the plates were washed three times with
DPBS. The plates were incubated at room temperature
05 with the primary antibody, Y1.2, at a 1:500 dilution
in 3% BSA in DPBS. The first antibody was then
removed and the plates were washed three times with
DPBS. The second antibody (.5ml), an alkaline-
phosphatase-conjugated goat anti-mouse IgG, was
10 diluted 1:1000 in 3% BSA in DPBS was added to the
plate which was incubated for 1 hr at room tempera-
ture. The second antibody was removed and the cells
were washed twice with DPBS and once with TBS. In
order to develop color on those plaques expressing
15 the leprosy 65Kd antigen, a BCIP/NBT mix (Kirkegaard
and Perry) was added to each plate. Plaques which
were positive for antigen expresion were picked with
a sterile toothpick into 2 ml of DME-2% FCS.

Enzyme-Linked Immunosorbance Assay (ELISA):
20   CV-1 cells were infected at an MOI of 2. The
cells were grown to confluence and infected as
described in the previous section, except that the
0.5ml of medium containing virus was left on the
cells after the 30 minute incubation, and 4.5ml of
25 fresh medium was added. The cells were then incu-
bated for 18 hours at 37°C.
        Following the incubation period, the medium was
aspirated from the cell monolayer, and the cells
were gently scraped off the dish into 1ml of DPBS.

This mixture was frozen and thawed (-80°C to 37°C) three times, and then sonicated. The treated cell pellets were used to coat microtiter plates to supply antigen on a solid phase for ELISA.

05   Immulon II remove-a-well strips (Dynatech) were coated in duplicate with 200ul of the cell pellet for 18 hrs at room temperature. The solution was then removed by aspiration, and 200ul of primary antibody (e.g. Y1-2 monoclonal diluted 1:500) in 50%

10 NGS was added to the wells. A negative control antibody, A_4E_7, was also used. This monoclonal antibody (in ascites fluid) recognized an epitope on the unrelated canine parvovirus. The primary antibody was incubated for six hr at 37°C. This

15 solution was removed by aspiration and the wells were washed three times with 0.5ml of washing buffer (0.05% Tween-20 in PBS, pH7.5). The secondary antibody was a horseradish peroxidase-conjugated goat anti-mouse IgG (heavy and light chain specific;

20 Jackson Immunoresearch) diluted 1:2,500 in 10% NGS. It was incubated at 200ul per well for 1 hr at 37°C. Following the incubation, the solution was removed by aspiration and the wells were washed three times, as described above. Color was developed using 3,3',

25 5,5'-tetramethyl-benzidine (TMB,Sigma) as the chromagen. Ten milligrams of TMB were dissolved in 1ml of DMSO, and 100ul of this solution was added to 5ml of acetate/citrate buffer (0.1M sodium acetate, pH6.0 with 0.1 M citric acid) along with 10ul of 3%

30 H_2O_2 (Park-Davis); 200ul of this final solution was

added to the wells and the plate was incubated at
room temperature for 5 min. The reaction was
stopped by the addition of 200ul of 2.5 N H_2SO_4.
One half of the solution in each well was removed,.
05 and the remaining 200ul was read at 450nm on the
Dynatech Mini-Reader II plate reader.

Metabolic Labeling:

CV-1 cells were grown for 24 hr to a density of
10^6 cells per 6cm plate and then infected with
10 vaccinia virus at an MOI of 2 for 30 min at 37°C.
Four hours later, the cells were labeled with
[^{35}S]methionine. The labeling medium consisted of
2ml of methionine-free DME, 3.5% FCS, 2mM L-glu-
tamine, 100uCi [^{35}S]methionine (New England Nuclear)
15 and carrier methionine (0.3mg/100ml). Alter-
natively, cells were labelled with [^{3}H] leucine (New
England Nuclear) in 2 ml of leucine-free DME, 3.5%
FCS, 2mM L-glutamine, approximately 100 uCi [^{3}H]
leucine and carrier leucine (1.05 ug/ml). Cells
20 were harvested after approximately 20 hr, washed
twice with PBS, and lysed with 0.5ml of immuno-
precipitation buffer (IPB: 10mM Tris-HCl, pH7.2,
650mM NaCl, 1% Triton X-100, 1% sodium deoxycholate,
0.1% SDS, 5mM EDTA, 1mM phenylmethylsulfonyl-
25 fluoride, and 0.1mg/ml trypsin inhibitor) for 10 min
at room temperature. For subsequent immuno-
precipitation steps, 1mM ATP was also included in
the buffer used. The lysates were stored at -80°C.

Preparation of Staphylococcus Aureus:

Killed and formalin-fixed S. aureus (IgSorb)
were obtained as a 10% (w/v) suspension from the
Enzyme Center (Malden, Ma). The cells were washed
05 twice with IPB and pelleted by spinning 5 min in a
microfuge. The cells were resuspended to a final
concentration of 10% or 20% (w/v) in IPB.

Preformed complexes of S. aureus and IgG were
prepared by incubating five volumes of 10% (w/v) S.
10 aureus with one volume of anti-vaccinia virus serum
for 10 min at 0°C. The complexes were pelleted,
washed twice, and resuspended to a final concen-
tration of 20% (w/v) in IPB.

Immunoprecipitation:
15   Immunoprecipitations were carried out on cell
lysate samples each containing 10uCi [^{35}S]methionine
in 0.2ml of IPB. All incubations with antibodies or
S. aureus were done with rocking at 4°C. Each
sample was precleared by incubating with 50ul of
20 performed immune complex (S. aureus-IgG, 20% [w/v])
for 1 hr and then clarifying by centrifugation in
the microfuge for 5 min. The supernatant was trans-
ferred to a new test tube, and the preclearing was
repeated. Each of the pellets was saved and pro-
25 cessed as described below. The resulting super-
natant was then treated for another 30 min with 50ul
of 20% (w/v) S. aureus. Finally, the supernatant
was transferred to a new test tube and incubated
with primary antibodies at an appropriate dilution.

The next day, 50ul of 10% (w/v) S. aureus were added
to the sample, and the incubation was continued for
30 min. The S. aureus complexes were pelleted by
centrifugation. Each pellet (including those from
05 the preclearing steps) was washed with 200ul of IPB.
The suspension was layered over a differential
sucrose gradient (400ul of 10% (w/v) sucrose/0.5 M
NaCl/IPB, plus 800ul of 30% (w/v) sucrose/0.14 M
NaCl/IPB) and spun for 10 min in the microfuge. The
10 resulting precipitate was washed with 200ul of IPB
and resuspended with 20ul of SDS gel sample buffer
(Laemmli, Nature 227; 680 (1970)). The sample was
boiled for 5 min, centrifuged to remove the
precipitate, and removed with a syringe for appli-
15 cation onto an SDS gel (Laemmli, U.K. 1970, Nature
227, 680) which contained an 8% separation gel and a
3% stacking gel. The SDS-polyacrylamide gel
electrophoresis was carried out under reducing
conditions and was followed by autoradiography of
20 the gel.

Immunoprecipitations Using Protein A-Sepharose in
Place of Preformed Complexes of S. Aureus - IgG:
^{3}H leucine - labeled lysates were precipitated
with trichloroacetic acid (TCA) to determine the
25 amount of radioactivity in each lysate. Antiserum
was added to an aliquot (1 uCi of TCA-precipitable
counts) of each sample and this mixture was then
gently agitated at 4°C for 2 hr to overnight.
Protein A-Sepharose was pre-swollen, washed twice

with IPB and 50 ul in IPB (1:1;v/v) was added and
the mixture was gently agitated for 1 hr at 4°C.
The Protein A-Sepharose was washed 4 times at 4°C
with 1 ml of IPB by spinning for 15 sec in a
05 microfuge to pellet the Protein A-Sepharose. The
Sepharose was then washed once with 1 ml of 0.01 M
Tris-HCl, pH8.2, 150 mM NaCl at 4°C. The pellets
were dried and resuspended in 20-40 ul of 1X Laemmli
sample buffer and prepared for analysis on SDS
10 polyacrylamide gels.

Immunization of mice:
    Adult female Balb/c mice (Taconic Labs) were
immunized intraperitoneally with 10 PFU of vaccinia
recombinants expressing mycobacterial antigens.
15   Serum was obtained from the vetro-orbital
plexus on a weekly basis for four weeks and was
assayed for the presence of anti-mycobacterial
antibodies on nitrocellulose filter lifts of
recombinant lambda phage.

20 Adsorption of mouse antiserum with an E. coli
lysate:
    In order to remove nonspecific binding of mouse
antisera to E.coli proteins, mouse antisera was
adsorbed on an E.coli lysate coupled to Affi-Gel
25 (Bio-Rad).
    A lysate of E.coli strain Y1090r-m$^+$ was made by
growing a one liter culture at 37°C to an A_{600} of
approximately 0.5. The bacteria were pelleted by

centrifugation at 4200 Xg for 10 min at 15°C. The
supernatant was discarded and the pellet resuspended
in 20 ml of PBS. This was frozen in liquid nitrogen
and then thawed at room temperature to generate the
05 lysate. The preparation was sonicated for 30
seconds and 7mls was dialyzed (in a 3500 molecular
weight cut-off membrane) against 1 liter of 0.01 M
bicarbonate buffer, pH 9.6, with three changes of
buffer.
10   Ten mls of both Affi-Gel 10 and Affi-Gel 15
(Bio-Rad) were mixed and were washed with 5 volumes
of ice-cold H_2O in a scintered glass funnel. This
gel was then added to the dialyzed lysate and the
mixture was incubated at room temperature for three
15 hours with constant gentle agitation. The gel was
then pelleted by centrifugation, and washed three
times with TBS. The gel was stored in an equal
volume of 0.002% NaN_3 in TBS.
         Mouse antisera was cleared of anti-E.coli
20 reactivity by incubating 0.1ml of mouse antiserum
plus 0.9 ml of 10% NGS with 0.4 ml of the
immunoadsorbent described above. It was incubated
with gentle mixing overnight at 4°C, and the
immunoadsorbent pellet was then removed by
25 centrifugation. This pre-cleared mouse serum was
then tested for antibody to mycobacterial antigens
on nitrocellulose filter lifts.

Screening Antisera on recombinant lambda phage:

Y1090r-m+ was grown overnight to saturation in
LB which contained 100ug/ml ampicillin. Fifty
microliters (200 PFU) of recombinant phage diluted
05 in 10mM Tris-HCl, pH7.5, 10mM $MgCl_2$, was added to
100ul of the Y1090 culture. This was incubated for
20 min at 37°C to allow phage to adsorb to the
bacteria. At this time 1.25 ml of soft agar
equilibrated at 48°C was added to each tube
10 containing the bacteria and phage. The tubes were
swirled gently and poured onto 6cm LB agar plates.
The cultures were incubated for 3.5 hr at 42°C.
They were then overlayed with nitrocellulose filters
saturated in IPTG, and incubated for 3.5 hours at
15 37°. The filters were carefully removed from the
dishes, and were blocked overnight at 4°C in 10% NGS
in PBS.

The blocking solution was aspirated, and
adsorbed mouse antiserum, diluted 1:10 in 50% NGS
20 was added and incubated for 1 hr at room
temperature. The filters were washed three times in
washing buffer (20mM Tris-HCl, pH7.5, 0.05% Tween,
1M NaCl) and were then incubated with alkaline
phosphatase labelled goat anti-mouse IgG
25 (Kirkegaard and Perry Labs) diluted 1:1000 in 10%
NGS. After 1 hr at room temperature, the filters
were again washed, and were then incubated with a
BCIP/NBT substrate solution (Kirkegaard and Perry
Labs) according to manufacturers instructions.

EXAMPLE 1

Construction of Plasmids pAbT2008 and pAbT6000
(Figure 1)

        pUC8 (Vieira and Messing, Gene 19:259 (1982))
05 was partially digested with HaeII, and the 2,258
base pair (bp) fragment was gel-purified and
religated to create pAG3 as shown in Figure 1A.
        pAbT2008 was a modification of pAG3 in that it
contained an EcoRI site at what was once the NdeI
10 site. It was created by digesting pAG3 with NdeI,
filling in the staggered DNA ends with Klenow
enzyme, ligating on EcoRI linkers (d(pGGAATTCC); New
England BioLabs), removing excess linkers by EcoRI
digestion, and religating the resulting DNA fragment
15 (Figure 1B). pAbT2008 was grown in the E. coli
strain JM101.
        The gene encoding the 65Kd antigen of
Mycobacterium leprae was contained within an EcoRI
DNA fragment cloned into lambda gt11. This lambda
20 clone was originally obtained from Dr. R. Young
(Whitehead Institute for Biomedical Research, Cam-
bridge, MA) and was designated lambda Y3178 (Young
et al., Nature 316:450 (1985)). This DNA was
digested with EcoRI, and the 3.6 Kb EcoRI DNA
25 fragment containing the M. leprae gene was purified
from an agarose gel. It was ligated to plasmid
pAbT2008 which had been digested with EcoRI and
treated with CIP, resulting in plasmid pAbT6000
(Figure 1C). This DNA was transformed into E. coli

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strain GM119, a dam⁻ E. coli strain, to yield
unmethylated plasmid DNA.

EXAMPLE 2

Construction of pDP502 (Figure 2):

05   Plasmids pMC1871 and pSKS107 (Shapira et al.,
Gene 25:71 (1983)) were obtained from M. Casadaban
(The University of Chicago; Chicago, Ill.) These
plasmids contained the E. coli lacZ gene flanked by
various restriction endonuclease sites.

10   Preparative amounts of pMC1871 were digested to
completion with SstI (SacI). Approximately 10ug of
this DNA was partially digested with BamHI and the
fragments were separated on a preparative agarose
gel. A 5,380bp band was isolated from this gel.

15 This fragment was missing the 5' end of the lacZ
gene from the BamHI site to the unique SacI site
found within the lacZ gene (Figure 2A).

          Preparative amounts of pSKS107 were digested to
completion with SacI and BamHI. The DNA fragments

20 were then separated on an agarose gel and a 2,040bp
fragment was isolated. This fragment contained the
5' end of the lacZ gene from a BamHI site to the
SacI site (Figure 2A).

          Appropriate quantities of these fragments

25 (5,380bp and 2,040bp) were then ligated to each
other using standard ligation conditions. The
resulting ligated DNA was used to transform

competent E. coli cells, strain RR1. This plasmid
was designated pMC1871-7 (Figure 2B)

05   Preparative amounts of pMC1871-7 were digested
with BamHI to completion, the fragments were then
separated on an agarose gel and a fragment of
approximately 3000bp was isolated. This fragment
contained the entire coding region of the lacZ gene
except for the five 5' most codons which were
deleted in the original pSKS107 construction (a

10 polylinker from a pUC plasmid was inserted in place
of these sequences in the original construction).
The 5' end of the lacZ gene had the following
predicted sequence: GA TCC GTC GAC CTG CAG CCA AGC
TTG GCA. The last amino acid codon GCA coded for

15 alanine which was the sixth amino acid in the native
lacZ polypeptide.

      Plasmid pRW120 (Panicali et al., Proc. Natl.
Acad. Sci. USA. 80:5364 (1983)) contained a PstI
fragment subcloned from the HindIII F fragment of

20 vaccinia virus (strain WR) DNA; the PstI fragment
spanned a unique BamHI site in the HindIII F frag-
ment. The PstI fragment was inserted into the
plasmid vector pBR325 (Bolivar et al., Gene 2:95
(1977)) from which the vector BamHI site had been

25 removed. The vaccinia BamHI site was adjacent to a
vaccinia transcription promoter region which had
been used to direct the expression of several
foreign genes inserted at the BamHI site. These
foreign genes were inserted such that their orien-

30 tations were consistent (in the same relative

orientation for transcription) with the vaccinia
gene.

A preparative amount of pRW120 was digested to
completion with BamHI and then treated with CIP.
05 This DNA was ligated to the BamHI lacZ fragment from
pMC1871-7 and the resulting DNA was used to trans-
form competent E. coli RR1 cells. This plasmid was
designated pDP502 (Figure 2C).

To determine if the coding region of the lacZ
10 gene was in frame with the vaccinia gene at the
BamHI site, pDP502 was sequenced through the junc-
tion of the 5' end of the lacZ insert and the
adjacent vaccinia DNA. This was done using standard
dideoxy sequencing techniques (Wallace et al., Gene
15 16:21 (1981)) directly on pDP502, using a commer-
cially available kit and M13 lacZ sequencing primer
(Amersham). The sequence analysis indicated that
the lacZ gene was in frame with the ATG of the
vaccinia promoter sequence (Figure 2D), allowing for
20 expression of B-galactosidase.

EXAMPLE 3

Construction of Plasmids pAbT400, pAbT401 and
pAbT402 (Figure 3):

pAG3 was digested with NdeI and treated with
25 the large fragment of DNA polymerase (Klenow) to
blunt the ends of the fragment. The vaccinia
thymidine kinase gene, located on the vaccinia
HindIII J fragment (Weir et al., Proc. Natl. Acad.

Sci. *USA*. *79*:1210 (1982); Hruby and Ball, *J*. *Virol*.
43:403 (1982); Weir and Moss, *J*. *Virol*. 46:530
(1983)), was subcloned into pBR322. The thymidine
kinase gene was excised from this clone by digesting
05   with HindIII and PvuII, producing an 1,800bp
fragment which was gel-purified and treated with
Klenow to blunt the ends of the fragment. The two
fragments described above were ligated to create
pAbT400, in *E*. *coli* strain MC1060, as shown in
10   Figure 3B.

pAbT400 was digested with ClaI and EcoRI and
was treated with CIP. The vaccinia 11K gene was
located on the vaccinia HindIII F fragment (Wittek
et al., *J*. *Virol*, *49*:371 (1984); Bertholet *et al*.,
15   *Proc*. *Natl*. *Acad*. *Sci*. *USA* 82:2096 (1985)), which
had been subcloned into pBR322 (Panicali and
Paoletti, *Proc*. *Natl*. *Acad*. *Sci*. *USA* *79*:4927
(1982)). This plasmid was digested with ClaI and
EcoRI, producing a 600bp fragment which was
20   gel-purified. The two fragments described above
were ligated to create pAbT401, in *E*. *coli* strain
MC1060, as shown in Figure 3C.

pAbT401 was digested with EcoRI and treated
with Klenow to produce a blunt-ended fragment;
25   BglII linkers (d(pCAGATCTG); New England BioLabs)
were ligated to the ends of the fragment and excess
linkers were removed by BglII digestion. pDP502 was
first digested with BglII and then partially di-
gested with BamHI; the resulting 3,900bp BglIIBamHI
30   fragment was gel-purified. The two fragments

described above were ligated to create pAbT402, in
E. coli strain MC1060, as shown in Figure 3D.

EXAMPLE 4

Construction of Plasmid pAbT6007 (Figure 4):

05   A 2,353bp DNA fragment containing the coding
region for the M. leprae 65Kd antigen was excised
from pAbT6000 by digestion with NruI and EcoRV.
BglII linkers (d(pGGAAGATCTTCC); New England Bio-
Labs) were ligated onto this DNA, excess linkers

10   were removed by BglII digestion, and the approxi-
mately 2,360bp DNA fragment was isolated from a gel
(Figure 4A).
     The plasmid pAbT402 was digested with BglII,
treated with CIP and then ligated to the 2,360bp DNA

15   fragment. This DNA was used to transform E. coli
JM101, and the resulting recombinant plasmid DNA
containing the M. leprae 65Kd gene linked to the
vaccinia 11K late promoter was designated pAbT6007
(figure 4B). This plasmid also contained the E.

20   coli lacZ gene regulated by the vaccinia BamF
(Panicali and Paoletti, Proc. Natl. Acad. Sci. USA,
79:4927 (1982); Panicali et al., Proc. Natl. Acad.
Sci. USA. 80:5364 (1983)) promoter. Flanking the
lacZ gene and the M. leprae gene were vaccina

25   thymidine kinase sequences. Cesium chloride-puri-
fied pAbT6007 DNA was prepared for IVR into vaccinia
virus strain NYCBH in CV-1 cells.

It should be noted that there was a translation
initiation codon (ATG) on the DNA fragment con-
taining the 11K promoter. Because the BglII linker
added onto the 2,360bp DNA fragment put this ATG in
05   the same reading frame as the translation initiation
codon (GTG) of the M. leprae 65Kd protein, the
recombinant vaccinia virus containing this DNA
should express a fusion protein that has its first
nine amino acids specified by the nucleotide se-
10   quence from the 11K promoter, the BglII linker and
those M. leprae nucleotides which precede its first
GTG (Figure 5). In addition, the GTG is expressed
as a valine in vaccinia whereas in M. leprae it is
the translation initiation codon and thus is
15   expressed as methionine.

EXAMPLE 5

Recombinant Vaccinia Virus Containing the leprosy
65Kd Antigen by In Vivo Recombination:
       In vivo recombination is a method whereby
20   recombinant vaccinia viruses are created (Nakano et
al., Proc. Natl. Acad. Sci. USA. 79: 1593 (1982)).
These recombinant viruses are formed by transfecting
DNA containing a gene of interest into cells which
have been infected by wild type vaccinia virus (in
25   this case NYCBH). The genes that one wants recom-
bined into the vaccinia genome are flanked on both
sides by vaccinia DNA sequences which serve to
direct the recombination of exogenous DNA to the

site where these vaccine sequences are found. A
small percent of the progeny virus will contain the
gene of interest integrated into a specific site on
the vaccinia genome. These recombinant viruses can
05 express genes of foreign origin (Panicali and
Paoletti, Proc. Natl. Acad. Sci. USA, 79: 4927
(1982); Panicali et al., 1983, Proc. Natl. Acad.
Sci, USA, 80: 5364 (1983)).

pAbT6007 DNA was transfected into vaccinia
10 virus-infected CV-1 cells at an MOI of 2. The virus
strain was NYCBH and the selection system for
recombinant virus was the appearance of blue plaques
due to the metabolism of Bluo-Gal directed by the
lacZ gene. Approximately 1×10^6 CV-1 cells on a 6cm
15 plate were infected for 40 min at 37°C. After
adsorption, 3.3ml of MEM-2% FCS was added to the
plate. For the calcium phosphate precipitation of
DNA, 243.2ul of Buffer A was added to 20ug of
pAbT6007 DNA (originally at a concentration of
20 2.95mg/ml), followed by the addition of 250ul of
Buffer B, as already described. After 40 min, this
DNA was added dropwise to the infected cells, which
were then incubated at 37°C until 100% of the cells
were observed to be infected.

25 The infected cells and virus were harvested and
the virus was titered as previously detailed. The
10^{-2} dilution resulted in 250-300 plaques per plate
and twenty 6cm plates of CV-1 cells were each
infected with 0.5ml of the 10^{-2} dilution of virus
30 obtained from the IVR. Several blue plaques were

picked from these plates, and after four cycles of
plaque purification, four final blue plaques were
picked and amplified twice on 6cm CV-1 plates. Two
of these were amplified on 15cm plates and

05   concentrated by centrifugation through a 36% sucrose
cushion as already described. One isolate,
vAbT53-1-1-1-2 was picked for further amplification
in a HeLa-S3 spinner culture. It was designated
vAbT53. The virus was centrifuged through a 25-40%

10   sucrose gradient in a SW28 rotor at 15,000 rpm at
4°C for 40 min. This preparation yielded virus at a
final concentration of 1.36×10^9 PFU/ml.

EXAMPLE 6

Black Plaque Assay for the Leprosy 65Kd Antigen
15 Expression:
     The black plaque assay is an _in situ_ enzyme-
based immunoassay which can detect protein expressed
by cells, both uninfected and those infected by
virus.

20   The black plaque assay was performed on the
recombinant vaccinia vAbT53, which contained the
gene for the leprosy 65Kd antigen, as well as on
wild-type vaccinia lacking this gene. Monoclonal
antibodies IIC8 or Y1-2 were used.

25   Plaques formed by the negative control, NYCBH
virus, showed only a background color which was
consistent with the background on the cell monolayer
itself. Plaques formed by the vaccinia recombinant

however, stained a distinct dark purple color which
was much darker than the background on the cell
monolayer. These results were obtained with both
antibodies, and showed that the recombinant virus
05 vAbT53 was expressing the M. leprae antigen.

                          EXAMPLE 7

Enzyme-Linked Immunosorbance Assay (ELISA) for the
Leprosy 65Kd Antigen Expression:
        The results are summarized in Table 1; mono-
10 clonal antibody Y1-2 recognized an antigen in vAbT53
but not in the uninfected cells or in cells infected
with NYCBH virus. The monoclonal A_4E_7 did not
recognize antigens in infected or uninfected cells.
These results showed that vABT53 was expressing the
15 M. leprae 65Kd antigen.

TABLE 1

Results* of ELISA on Vaccinia Recombinant vAbT53 Expressing the 65Kd M. Leprae Antigen.

Monoclonal Antibody	Specificity	Cells Infected with:		
		vAbT53	NYCBH	No Infection
Y1-2	65 kd antigen of M. leprae	1.28	0.60	0.49
A_4E_7	canine parvo-virus	0.13	0.12	0.13

* . Results are expressed as the average of duplicate
O.D. readings at 450nm on the ELISA plate reader.

EXAMPLE 8

Immunoprecipitation of Leprosy 65Kd Antigen From
Vaccinia Recombinant vAbT53:

        Immunoprecipitation analysis was performed on
05 CV-1 cells infected with recombinant vaccinia virus
    vAbT53 encoding the 65Kd protein of M. leprae.
    Cells were labeled with [^{35}S]methionine for approxi-
    mately 20 hr. The cell lysates were precleared of
    background proteins using an anti-vaccinia virus
10 serum and then incubated with a mouse monoclonal
    antibody IIC8 (dilution 1:200) directed against the
    65Kd protein. Antibodies were precipitated with S.
    aureus, and the crossreacting antigens were detected
    by SDS-polyacrylamide gel electrophoresis followed
15 by autoradiography.
        Each of the three vaccinia recombinant samples
    tested incorporated radioactivity into a 68Kd
    protein. This signal was absent in cells infected
    with wild-type vaccinia virus or with recombinant
20 virus containing other foreign genes. Background
    proteins that were derived from vaccinia-encoded
    genes were identified on the basis of their cross-
    reactivity with the anti-vaccinia virus serum.

EXAMPLE 9

Construction of pAbT8000 and pAbT8005 (Figure 6)

Lambda phage Y3253 (Husson and Young, Proc.
Natl. Acad. Sci. USA 84:1679 (1987)) which contained
05 the gene encoding the tuberculosis 65kd antigen, was
obtained from Dr. R. Young of the Whitehead
Institute of Biomedical Research, Cambridge, MA.
Phage DNA was prepared and then digested with EcoRI.
This EcoRI DNA fragment of approximately 2.1kb
10 contained the gene for the tuberculosis 65Kd antigen
and was purified from an agarose gel. It was
ligated to pEMBL18 DNA (Dente et al. Nuc. Acids Res.
11:1645 (1982)) which had been digested with EcoRI
and treated with CIP. The resulting plasmid, shown
15 in Figure 6, was designated pAbT8000.

DNA from pAbT8000 was then digested with the
restriction enzymes BspMII and KpnI. BspMII cleaves
the DNA just upstream of the initiating ATG codon.
The DNA was then blunt ended with T4 DNA polymerase.
20 BglII linkers (New England Biolabs #1052) were
ligated onto the blunt-ended DNA. The addition of
this BglII linker served to place the ATG contained
within the 11K promoter fragment into frame with the
ATG of the gene encoding the 65Kd antigen. The DNA
25 was digested with BglII and a fragment of about
1.8Kb was purified from a gel and ligated to pAbT402
DNA which had been cut with BglII and treated with
CIP. The ligated DNA was used to transform HB101
E.coli cells, and minipreparations of plasmid DNA

were purified and digested with various restrcition
enzymes to isolate a plasmid in which the gene for
the tuberculosis 65Kd antigen was in correct
orientation relative to the 11K promoter of pAbT402.

05   This resulting plasmid was designated pAbT8005
(Figure 6) and contains the vaccinia 11K promoter
driving the expression of the gene for the
tuberculosis 65Kd antigen. This 11K - 65Kd
cartridge is followed by the vaccinia Bam F promoter

10 controlling the expression of the lacZ gene. These
two promoters and their genes are flanked by the
vaccinia TK gene which serves to direct in vivo
homologous recombination into the vaccinia TK gene.

EXAMPLE 10

15 Construction of pAbT8008 (Figure 7)

       pAbT8005 DNA (Figure 7), which contains the
gene for the tuberculosis 65Kd antigen, was digested
with BglII and then blunt-ended with Klenow. A
fragment of approximately 1.8 Kb was then purified

20 from an agarose gel and ligated to pAbT4007 DNA
which had been digested with SmaI and then treated
with CIP. pAbT4007 is a vaccinia expression vector
(See U.S. Patent application Serial No. 910,501, -
Panicali, Mazzara & Gritz filed September 23, 1986)

25 which contains a 7.5K promoter to drive the
expression of an inserted foreign gene. As in
pAbT402, the pAbT4007 vector contains a downstream
vaccinia BamF promoter driving lacZ expression. The

ligation mixture consisting of pAbT4007 DNA and the
BglII fragment from pAbT8005 was used to transform
E. coli HB101 cells. Minipreparations of DNA were
purified and the plasmids were analyzed by re-
05 striction digests to determine which DNAs contained
the BglII fragment in the proper orientation for the
expression regulated by the 7.5K promoter. This DNA
was then designated pAbT8008.

   pAbT8008 contains the vaccinia thymidine kinase
10 gene flanking the following.
   1.   the vaccinia 7.5K promoter driving the
        expression of the gene for the
        tuberculosis 65Kd protein.
   2.   the Bam F promoter driving the expression
15   of the gene for lacZ.

                        EXAMPLE 11

Construction of pAbT8032 (Figure 8)
   DNA from pAbT6007 (Figure 4B) was digested with
BglII to generate a 2352bp fragment which contained
20 the coding sequence for the gene for the leprosy
65Kd protein. The DNA was then blunt-ended with
Klenow, and Cla linkers (New England Biolabs #1037)
were ligated on. The Cla linker provided an ATG
which was in frame with the coding sequence of this
25 leprosy gene, and was needed because the 7.5K
promoter lacked an ATG and the initiation codon on
this mycobacterial gene was GTG, instead of the
usual ATG. The DNA was then cleaved with ClaI and

fractionated on an agarose gel. The 2,360 fragment
was purified and ligated to pBR322 DNA that had been
digested with ClaI and treated with CIP. The
ligated DNA was transformed into E. coli HB101 and a
05 clone containing the ClaI insert was designated
pAbT8031 (Figure 8).

   pAbT8031 DNA was digested with ClaI and treated
with Klenow to blunt the ends. The DNA was
fractionated by size on an agarose gel, and the
10 2,360 bp fragment containing the gene for the
leprosy 65Kd antigen linked to an in-frame ATG was
ligated to pAbT4007 DNA which had been cut with SmaI
and then treated with CIP. A clone containing the
gene for the leprosy 65Kd antigen in the correction
15 orientation for expression regulated by the 7.5K
promoter was isolated and designated pAbT8032
(Figure 8).

   pAbT8032 contains the gene for the leprosy 65Kd
antigen preceded by an in-frame ATG which serves as
20 the initation codon. This gene is controlled by the
vaccinia 7.5K promoter and is followed by the BamF
promoter driving the expression of the lacZ gene.
These elements are then flanked by the vaccinia
thymidine kinase gene.

EXAMPLE 12

<u>Construction of pAbT8023, pAbT8502 and pAbT8501</u>
<u>(Figure 9)</u>

Lambda clone Y3184 (obtained from R. Young,
05 Whitehead Institute contains the gene encoding a
protein which exhibits an affinity to a
biotin-conjugated antibody for a leprosy 12Kd
antigen. The DNA encoding the antigen was sequenced
(R. Young, personal communication) and found to be
10 incomplete. The predicted amino acid sequence of
this protein has homology to several proteins which
are known to bind biotin (e.g. Maloy <u>et al</u>., <u>J</u>.
<u>Biol</u>. <u>Chem</u>. <u>254</u>:11615 (1979)) and it is possible
that this is the reason the lambda clones reacted
15 postively to the biotin-conjugated antibody. The
DNA sequence predicts a polypeptide whose minimum
size is 18Kd, and based upon the size of other
biotin-binding proteins, this may be close to the
full-length size. For purposes of continuity, this
20 DNA is still referred to as the gene for the leprosy
12Kd antigen.

DNA from lambda clone Y3184 was isolated and
digested with EcoRI. This EcoRI fragment was then
cloned into pEMBL18 DNA which had been digested with
25 EcoRI and treated with CIP. The ligated DNA was
transformed into <u>E</u>. <u>coli</u> strain HB101, and a plasmid
containing the 2.4Kb EcoRI insert was designated
pAbT8023 (Figure 9).

pAbT8023 DNA was digested with EcoRI and then blunted with Klenow. Cla linkers (New England Biolabs #1089) were ligated onto the blunt-ended DNA which was then digested with ClaI. Because this

05 leprosy gene was not a full-length sequence, an in-frame ATG was needed, and we have provided one with this Cla linker. The 620bp Cla-linkered fragment was purified from a gel and ligated with pBR322 DNA that had been cut with ClaI and treated

10 with CIP. The ligated DNA was transformed into HB101 cells, and minipreparations of DNA were prepared and analyzed by restriction digests for isolation of a clone which contained a 620bp ClaI DNA fragment. This clone was designated pABT8502

15 (Figure 9).

pAbT8502 DNA was digested with ClaI and then blunt-ended with Klenow. The 620bp fragment was cloned into the SmaI site of pAbT4007. This DNA ligation was transformed into HB101 cells. The DNA

20 prepared from various HB101 colonies was analyzed by restriction endonuclease analysis in order to isolate a plasmid which contained the sequence for the leprosy 12Kd antigen in the correct orientation for expression from the 7.5K promoter. This DNA was

25 designated pAbT8501 (Figure 9).

pAbT8501 contains the gene for the leprosy 12Kd antigen under the control of the 7.5K promoter and this cartridge is followed by the BamF promoter driving the lacZ gene. The vaccinia thymidine

30 kinase gene flanks these two foreign genes.

EXAMPLE 13

Recombinant Vaccinia Virus Containing the Leprosy
65Kd Antigen (vAbT121), Tuberculosis 65Kd Antigen
(vAbT86, vAbT88) or the Leprosy 12Kd Antigen
05 (vAbT117).

vAbT121 was created by infecting cells CV-1
cells with NYCBH at an MOI of 0.05 and transfecting
in 15 ug of linearized pAbT8032 (original
concentration of 1.33 mg/ml) DNA. The IVR was
10 harvested at 48 hr and then titered. The
recombinant was purified through four rounds of
plaque purification on BSC40 cells grown in DME. In
the first, third and fourth rounds, recombinant
plaques were picked by their blue phenotype in the
15 presence of Bluo-gal and IPTG. The determination of
recombinant plaques in the second round of
purification was done by the live black plaque assay
wherein the primary antibody was the Y1.2 mouse
monoclonal and the second antibody was an alkaline
20 phosphatase conjugated goat anti-mouse IgG. The
final isolate vAbT121C-B-1L-1-1 was designated
vAbT121 and amplified in a Hela-S3 spinner culture.
This virus expressed the leprosy 65Kd antigen during
early and late infection under the control of the
25 7.5K promoter.

vAbT86 which expresses the tuberculosis 65Kd
antigen under the control of the 11K promoter during
late infection, was produced by infecting CV-1 cells
with NYCBH at an MOI of 1, and then transfecting in
30 20 ug of pAbT8005 plasmid DNA (original

concentration 1.48 mg/ml). The virus from the IVR
was harvested after 24 hr and the recombinants were
purified through four rounds of plaque purification
on CV-1 cells. In each round, the expression of the
05 lacZ gene indicated the recombinant plaques. A
final isolate, vAbT86-2-2-1-1 was picked for further
amplication and designated vAbT86.

vAbT88 was generated by infecting CV-1 cells
with NYCBH at an MOI of 1, and transfecting in 20 ug
10 of pAbT8008 plasmid DNA (original concentration of
4.7 mg/ml). The virus resulting from the IVR was
harvested after 24 hr. Recombinants were purified
through four rounds of plaque purification by lacZ
expression on CV-1 cells. A final isolate,
15 vAbT88b-2-1-1-1, expressed the tuberculosis 65Kd
antigen during early and late infection, was
designated vAbT88. Expression of this antigen was
under the control of the 7.5K prompter.

vAbT117 was prepared by infecting CV-1 cells
20 with NYCBH and transfecting in 20 ug of pAbT8501
plasmid DMA (original concentration 2.9 mg/ml). The
virus resulting from this IVR was harvested at 48
hr and recombinants were purified through three
rounds of plaque purification on CV-1 cells.
25 Recombinants were selected by expression of the lacZ
gene at each round of purification. One isolate,
vAbT117-18-1-4-1 was designated vAbT117 and was
amplified on Hela-S3 cells. The characteristics of
these four recombinant viruses are summarized in
30 Table 2. Results of immunoprecipitation and animal
studies are also tabulated here. The predicted

amino acid sequences encoded by these recombinant
virus are shown in Table 3. It is expected that
these proteins will not significantly differ from
the proteins produced in mycobacteria.

05   EXAMPLE 14

Black Plaque Assay for Tuberculosis and Leprosy 65Kd
Antigen Expression:
        vAbT86, and vAbT88, and vAbT121 were tested by
a black plaque assay for expression of the
10 tuberculosis 65Kd antigen with the Y1-2 monoclonal
antibody. NYCBH was used as a negative control.
Plaques formed by the three vaccinia recombinants
were stained a dark purple color while control
plaques showed only a background color. A
15 mycobacterial 65Kd antigen was being expressed by
each recombinant virus.

                         EXAMPLE 15

ELISA for the Tuberculosis and Leprosy 65Kd Antigen
Expression:
20   ELISA results for vAbT86, vAbT88 and vAbT121
are summarized in Table 4. Monoclonal antibodies
Y1-2 and TB-78 recognized antigens in cells infected
by these three recombinant viruses but not in
uninfected cells or cells infected by the wild-type
25 NYCBH virus. vAbT86 and vAbT88 were expressing the
tuberculosis 65Kd antigen while vAbT121 was
expressing the leprosy 65Kd antigen.

TABLE 2

SUMMARY OF RECOMBINANT VIRUSES AND THEIR IMMUNOGICAL RESULTS

Plasmid	Virus	Promoter	When Expressed	Antigen	ELISA Y1.2[2]	ELISA TB78[2]	Immunoprecipitations Strength and Molecular Weight Y1.2	Immunoprecipitations Strength and Molecular Weight TB78	Injected Into Mice	Anti 65K Antibody Detected
pABT8005 PTK11KTB65K502	vAbT86	11K	Late	TB65K	++	++	Good 66K	Very Faint 66K	Yes	Yes
pABT8008 PTK7.5KTB65K502	vAbT88	7.5K	Early & Late	TB65K	+	-	Strong 68K	Good 68K	Yes	Yes
pABT6007 PTK11KL65K502	vAbT53	11K	Late	Leprosy 65K	++	ND[4]	Good 68K	Negative	Yes	Yes
pABT8032 PTK7.5KL65K502	vAbT121	7.5K	Early & Late	Leprosy 65K	++	ND[4]	Good 68K	ND[4]	No	-
pABT8501 PTK7.5KL12K502	vAbT117	7.5K	Early & Late	Leprosy 12K	ND[4]	ND[4]	ND[4]	ND[4]	Yes	Yes[5]

Notes:

1. Don't know if this promoter is active during late infection.

2. Y1.2 monoclonal antibody recognizes leprosy 68K (L65K) antigen and is cross-reactive with the tuberculosis 65K (TB65K) antigen. The TB78 monoclonal antibody recognizes TB65K and is not cross-reactive.

3. Leprosy 12K expression also checked by RNA dot blot analysis and by hybrid selection.

4. Not done.

5. Antibodies in mice sera were tested by reaction with β-gal fusion proteins produced by lambda gt11.

TABLE 3

Predicted Amino Acid Sequences of Mycobacterial Proteins Encoded by Recombinant Vaccinia Viruses

Leprosy 65Kd Antigen:

```
Bacteria                                         Leprosy
                               C GAT CAG CGA GTG CCA GGT CGG
                                   D   Q   R   V   P   G   R

vAbT53             11K          Linker           Leprosy
(pAbT6007)   ATG AAT TCA | GAT CTT CCC | GAT CAG CGA GTG CCA GGT CGG
              M   N   S     D   L   P     D   Q   R   V   P   G   R

vAbT121      7.5K Linker   Linker            Leprosy
(pAbT8032)   CCC CCG ATG | GAT CTT CCC | GAT CAG CGA GTG CCA GGT CGG
                      M     D   L   P     D   Q   R   V   P   G   R
```

Tuberculosis 65Kd Antigen:

```
Bacteria                                              Tuberculosis
                             C CGG ACC AAT CAC TTC GCA ATG GCC AAG ACA
                                 R   T   N   H   F   A   M   A   K   T

vAbT86             11K          Linker                Tuberculosis
(pAbT8005)   ATG AAT TCA | GAT CTT CCC | CGG ACC AAT CAC TTC GCA ATG GCC AAG ACA
              M   N   S     D   L   P     R   T   N   H   F   A   M   A   K   T

vAbT88       7.5K Linker    Linker            Tuberculosis
(pAbt8008)   C CCC | GAT CTT CCC | CGG ACC AAT CAC TTC GCA ATG GCC AAG ACA
                     D   L   P     R   T   N   H   F   A   M   A   K   T
```

Leprosy 12Kd Antigen:

```
Bacteria                  Linker[1]         Leprosy
                        GAA TTC | GAG GTC GAA GGC CTG
                         E   F     E   V   E   G   L

vAbT117      7.5K Linkers            Leprosy
(pAbT8501)   CCC CCG ATG GAA TTC | GAG GTC GAA GGC CTG
                      M   E   F     E   V   E   G   L
```

Notes
1. Linkers used in constructing lambda gt11 library

TABLE 4

Results of ELISA on Vaccinia Recombinants vAbT86, vAbT88 and vAbT121.

ELISA[1]

05 Vaccina Recombinant	Antibody	Recombinant	NYCBH	No Infection
vAbT86	TB78	1.83	0.25	N.D.[2]
vAbT88	Y1.2	1.61	0.25	N.D.[2]
vAbT121	Y1.2	0.61	0.10	0.06

10 Notes:

1. Results are expressed as the average of duplicate O.D. readings at 450nm on the ELISA plate reader.

2. Not done.

EXAMPLE 16

<u>Immunoprecipitation of the Tuberculosis and Leprosy</u>
<u>65Kd Antigen:</u>
     CV-1 cells were infected with vAbT86, vAbT88,
05 vAbT121 or NYCBH. Cells were labeled with ^{3}H
leucine for approximately 20 hr. Cells were
harvested and immunoprecipitations were done as
described using protein A-Sepharose. The results
are summarized in Table 2. All three recombinants
10 express a 65 - 68Kd antigen which was not present in
NYCBH.

EXAMPLE 17

<u>Expression Analysis of the Leprosy 12Kd Antigen:</u>
     Because we were unable to obtain good
15 monoclonals against this particular antigen, the
expression analysis of vAbT 117 was accomplished by
RNA dot blots, hybrid selection and generation of
mouse antiserum to the original lambda gt11 clone
Y3184. The hybridization with RNA dot blots showed
20 that CV-1 cells infected with vAbT117 contained RNA
which hybridized to a 620bp ClaI DNA fragment
purified from pAbT8502. Hybrid selection showed
that RNA obtained from CV-1 cells infected with
vAbT117 hybridized to pAbT8502 plasmid DNA and this
25 RNA was translated into a polypeptide of
approximately 18-19 Kd. The RNA for the hybrid
selection experiments was obtained from CV-1 cells

that had been infected at an MOI of 10; these cells
were treated with cycloheximide, which was present
during the entire infection (Mahr and Robert, J.
Virol. 49:497 (1984).

05   Mice injected with the recombinant vAbT117 were
bled and their sera were tested for the presence of
antibodies to the lacZ fusion protein from Y3148.
The results showed that the mouse sera contained
antibodies to the polypeptide whose DNA sequence was
10 contained within Y3148. This polypeptide is the
12Kd antigen.

EXAMPLE 18

Mouse Antisera Raised to Leprosy and Tuberculosis
65Kd Antigen:

Mice were vaccinated with 10 PFU of vAbT86,
05   vAbT88 or vAbT53. The sera were collected at
various times post inoculation and then tested for
the presence of antibodies to the leprosy or
tuberculosis 65Kd antigen. Lambda gt11 clone Y3178
was used as the antigen source for vAbT53. Clone
10   Y3253 was the source of tuberculosis 65Kd antigen,
the target of mouse antibodies generated by
inoculating with vAbT86 and vAbT88. The results
showed that vAbT86 and vAbT88 were able to raise
mouse antibodies to the tuberculosis 65Kd antigen
15   synthesized by E.Coli infected with phage Y3253.
Vaccinia recombinant vAbT53 elicited mouse
antibodies to the polypeptide synthesized by E.coli
infected with Y3178.

Deposit of Biological Material
20   Plasmid pAbT6007 was placed on deposit at the
American Type Culture Collection (ATCC) in
Rockville, MD on September 5, 1986. Plasmids
pAbT8005, pAbT8008, pAbT8032 and pAbT8501 were
placed on deposit at the ATCC on September __, 1987.

25 Equivalents
Those skilled in the art will recognize, or be
able to ascertain using no more than routine experi-
mentation, many equivalents to the specific embodi-
ments of the invention described herein. Such

equivalents are intended to be encompassed by the
following claims.

CLAIMS

1. A recombinant pox virus capable of expressing a mycobacterial antigen.

2. A recombinant pox virus of Claim 1, which is
05   the species vaccinia.

3. A recombinant pox virus of Claim 1, wherein the mycobacterial antigen is an antigen of M. leprae, M. tuberculosis or M. bovis.

4. A recombinant pox virus of Claim 3, wherein the
10   antigen is the 65Kd antigen of M. leprae.

5. A recombinant pox virus of Claim 3 wherein the antigen is the 12Kd antigen of M. leprae.

6. A recombinant pox virus of Claim 3 wherein the antigen is the 65Kd antigen of M. tuberculosis.

15 7. A recombinant vaccinia virus capable of expressing the 65Kd antigen of M. leprae or M. tuberculosis.

8. A recombinant vaccina virus of Claim 7 selected from the group consisting of vAbT86, vAbT88,
20   vAbT53 and vAbT121.

9.   A recombinant vaccinia virus capable of
     expressing the 12Kd antigen of M. leprae.

10. A recombinant vaccinia virus of Claim 9
     comprising vABT117.

05 11. A recombinant pox virus capable of expressing a
     mycobacterial antigen which is capable of
     inducing immunity against mycobacteria.

12. A recombinant pox virus of Claim 11, which is
     of the species vaccinia.

10 13. A recombinant pox virus of Claim 11, wherein
     the mycobacterial antigen is an antigen of M.
     leprae, M. tuberculosis or M. bovis.

14. A recombinant pox virus of Claim 13, wherein
     the antigen is the 65Kd antigen.

15 15. A recombinant pox virus of Claim 11 wherein the
     antigen is the 12Kd antigen of M. leprae.

16. A plasmid DNA vector for in vivo recombination
     with a pox virus to produce a recombinant pox
     virus capable of expressing a mycobacterial
20   antigen, comprising;
     a.   structural gene or portion thereof en-
          coding a mycobacterial antigen;

b. a pox virus promoter linked to the structural gene and positioned so as to direct expression of the structural gene encoding the mycobacterial antigen;

05 c. DNA sequences of a pox virus flanking the promoter and structural gene at both 5' and 3' ends, the flanking pox sequences being sequences which allow recombination into a region of pox virus which does not

10 inhibit the ability of the virus to replicate.

17. A plasmid of Claim 16, wherein the structural gene encodes a mycobacterial antigen of M. leprae, M. tuberculosis or M. bovis.

15 18. A plasmid of Claim 16, wherein the pox virus is of the species vaccinia.

19. A plasmid of Claim 16, wherein the promoter is selected from the group consisting of the vaccinia 11K, 7.5K, 30K, 40K and BamF

20 promoters.

20. A plasmid of Claim 16, wherein the flanking sequences are derived from the gene of vaccinia virus which encodes thymidine kinase.

21. A plasmid of Claim 16, further comprising;

    d.   a second promoter of the pox virus linked
to a second structural gene which encodes
a marker or indicator for selection of
recombinant pox virus, the second promoter

05   and second structural gene being located
between the flanking DNA sequences of the
pox virus.

22.   A plasmid of Claim 21, wherein the second
promoter of the pox virus is the BamF promoter

10   of vaccinia.

23.   A plasmid of Claim 21, wherein structural gene
which encodes a selectable marker is the lacZ
gene.

24.   A plasmid DNA vector for in vivo recombination

15   with vaccinia virus to produce a recombinant
vaccinia virus capable of expressing a myco-
bacterial antigen, comprising;

    a.   structural gene or portion thereof en-
coding an antigen of M. leprae, M. tuber-

20   culosis or M. bovis;

    b.   a first vaccinia promoter linked to the
structural gene, the promoter being
located upstream with the structural gene;

    c.   a lacZ gene;

25   d.   a second vaccinia promoter linked to the
lac Z gene, the promoter being located

upstream of and in proper reading frame
with the lac Z gene; and

e.   DNA sequences flanking the construct of
elements a-d, the sequences being
05   homologous with sequences of the vaccinia
thymidine kinase gene.

25.   A plasmid DNA vector of Claim 24, wherein the
antigen is the 65Kd antigen.

26.   A plasmid DNA vector of Claim 24 wherein the
10   antigen is the 12Kd antigen of M. leprae.

27.   A plasmid vector of Claim 24, wherein the first
promoter is linked to the gene encoding the
mycobacterial antigen through a region of the
gene for the 11Kd antigen containing at least
15   the translational start signal.

28.   The plasmids pAbT8005, pAbT8008, pAbT6007,
pAbT8032 and pAbT8501.

29.   A method of preparing a recombinant pox virus
capable of expressing a mycobacterial antigen,
20   comprising the steps of:
a.   providing a plasmid DNA vector comprising:
i.   structural gene or portion thereof
encoding a mycobacterial antigen;
ii. a pox virus promoter linked to the
25   structural gene and positioned so as

                         to direct expression of the struc-
                         tural gene encoding the mycobacterial
                         antigen;
                  iii.   DNA sequences of a pox virus flanking
05   the promoter and structural gene at
                         both 5' and 3' ends, the flanking pox
                         sequences being sequences which allow
                         recombination into a region of pox
                         virus which does not inhibit the
10   ability of the virus to replicate;
            b.   allowing the plasmid vector to recombine
                  with pox virus _in vivo_ to produce
                  recombinant pox virus; and
            c.   isolating the recombinant pox virus.

15   30.   A method of Claim 29, wherein the pox virus is
            a vaccinia virus.

      31.   A method of Claim 29, wherein the structural
            gene encodes a mycobacterial antigen of M.
            leprae, M. tuberculosis or M. bovis.

20   32.   A method of Claim 29, wherein the structural
            gene encodes the 65Kd antigen of M. leprae or
            M. tuberculosis.

      33.   A method of Claim 29, wherein the structural
            gene encodes the 12Kd antigen of M. leprae.

34. A method of Claim 29, wherein the promoter is the vaccinia 11K, 7.5K, 30K, 40K or BamF promoter.

35. A method of Claim 29, wherein the step of allowing the plasmid vector to recombine with the pox virus _in_ _vivo_ comprises:

    i.   infecting the host cell with pox virus;

    ii. transfecting the infected cell with the plasmid vector; and

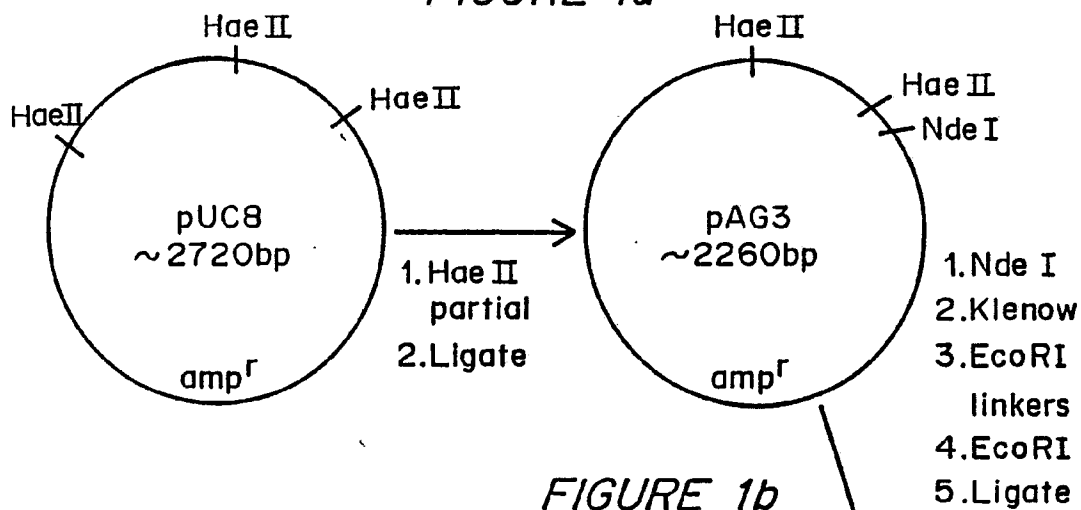   iii. incubating the cells under conditions which allow the virus to replicate.
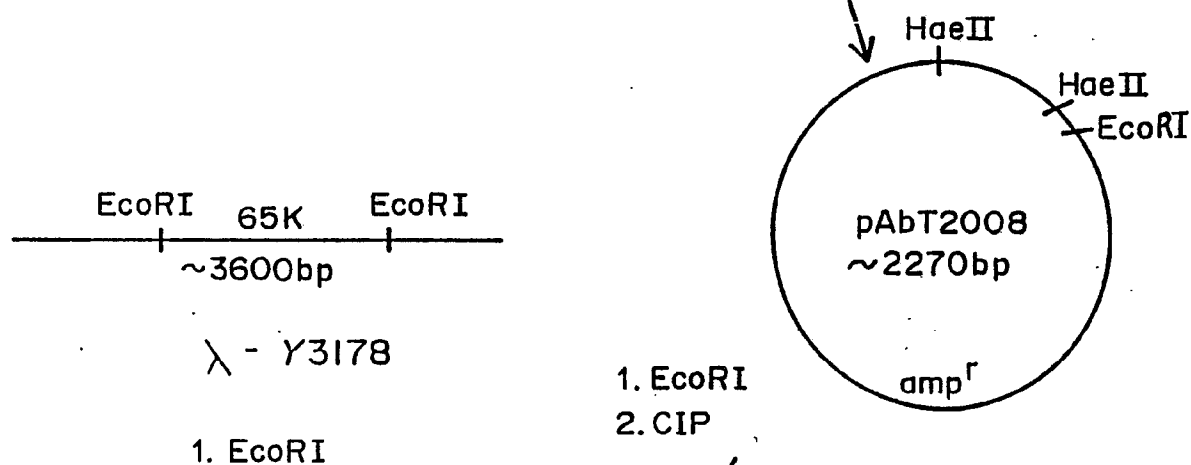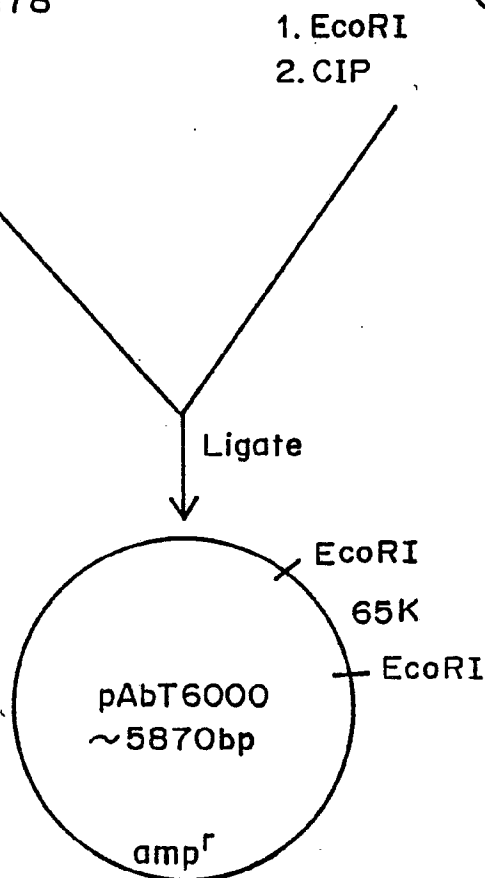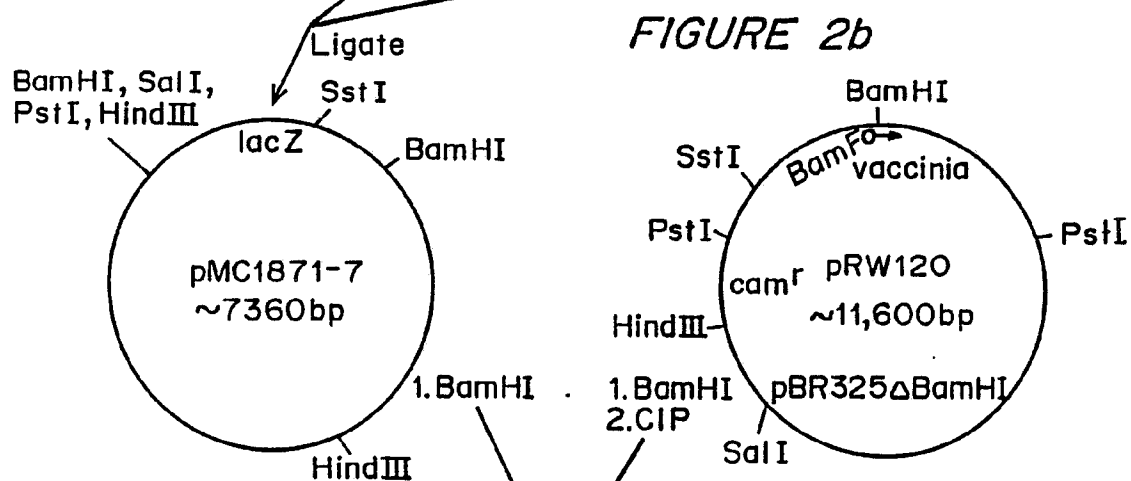
FIGURE 1a

FIGURE 1b

FIGURE 1c
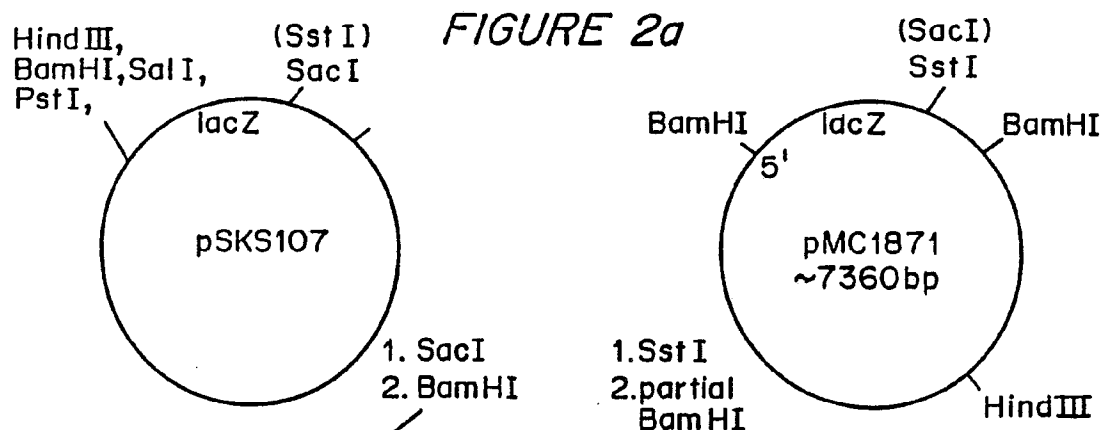
FIGURE 2a
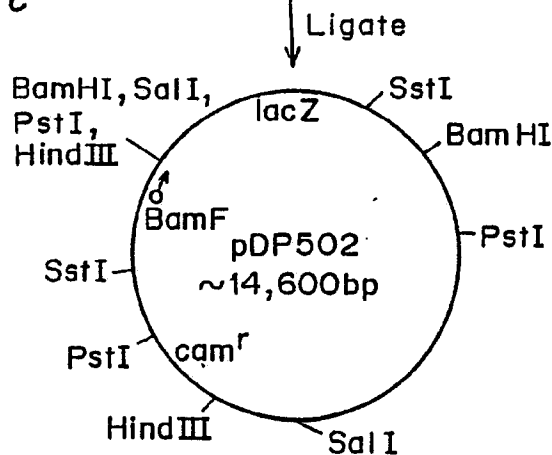
FIGURE 2b

FIGURE 2c

FIGURE 2d

Sequence at junction of vaccinia BamF and lacZ:
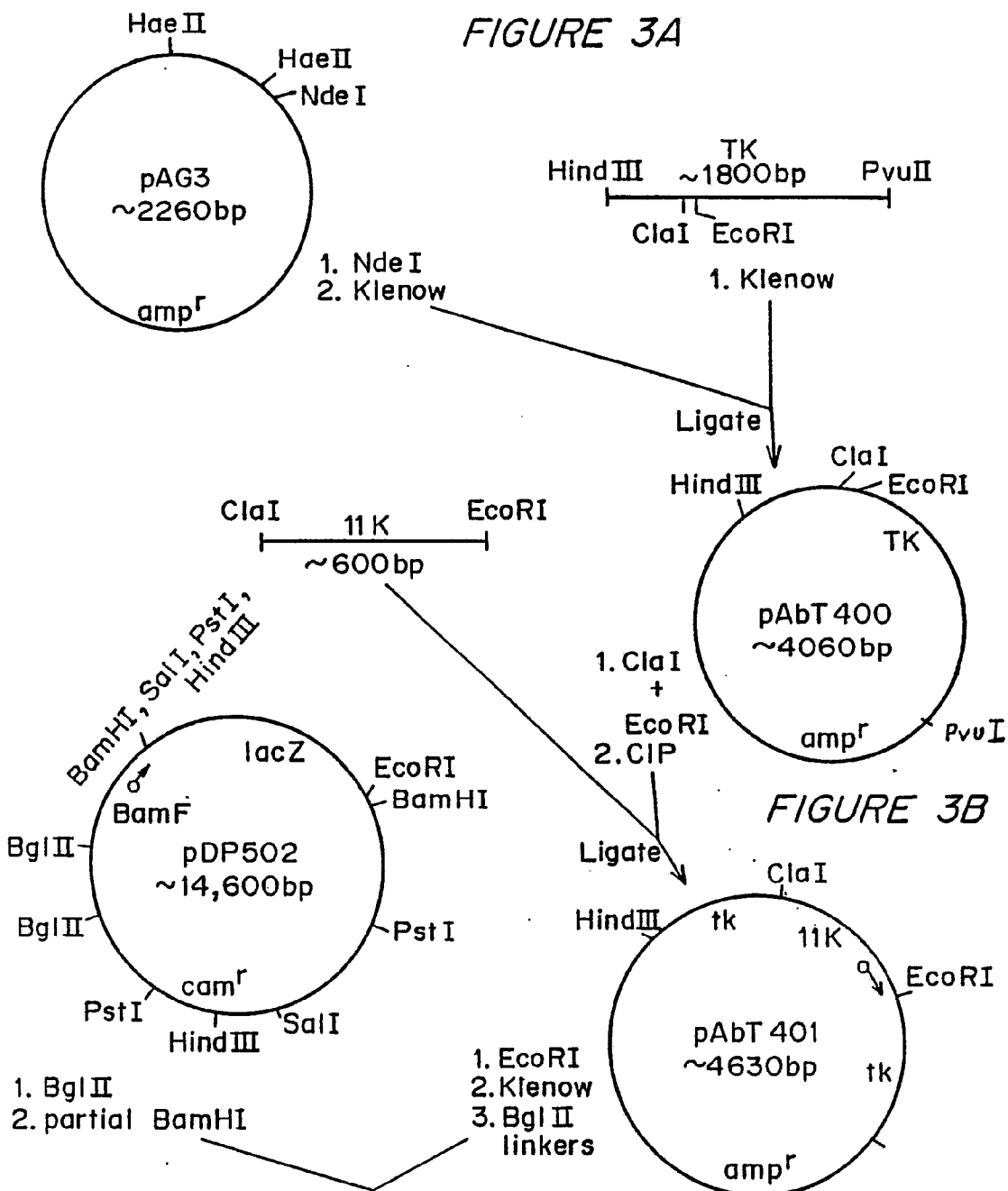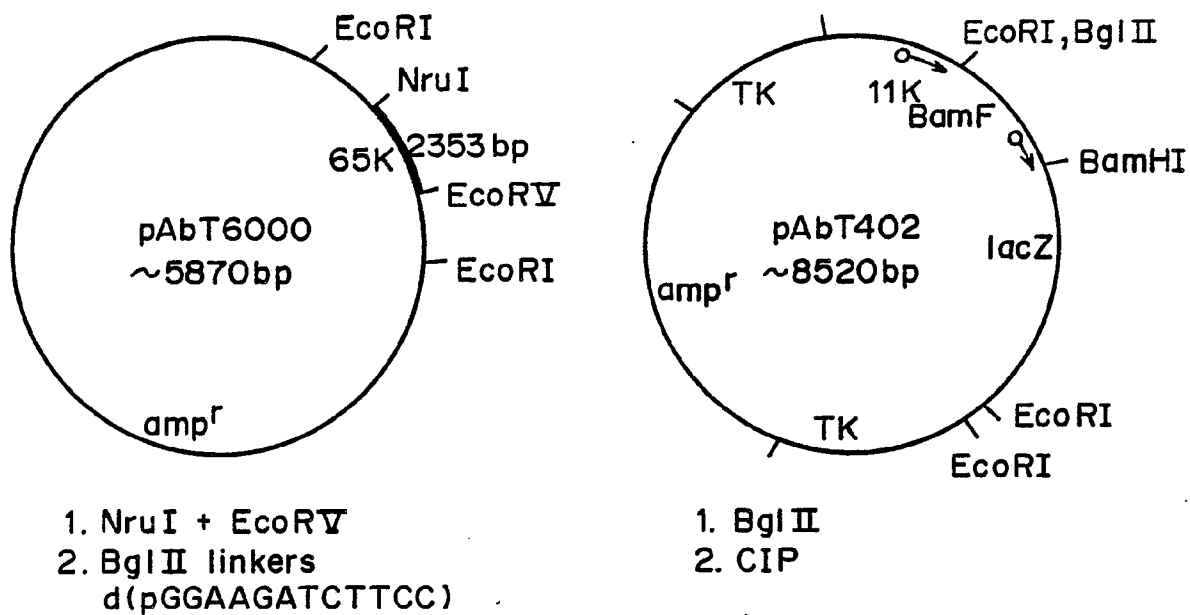T AAT ATG ACG CTC GTC ATG GGA TCC GTC GAC CTG CAG CCA AGC TTG GCA

FIGURE 3A

FIGURE 3B

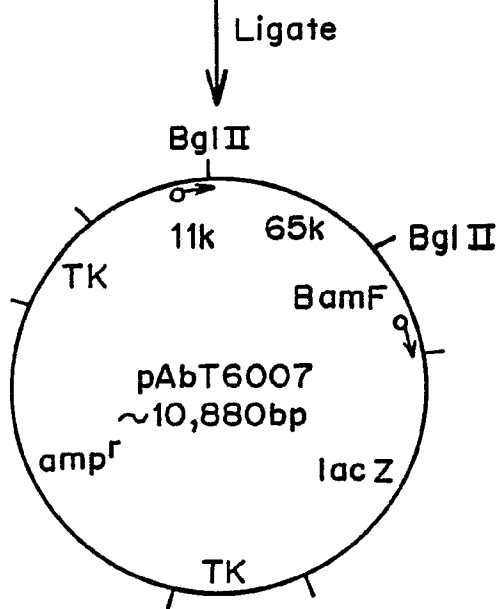FIGURE 3C

FIGURE 4a

pAbT6000
~5870 bp

EcoRI
NruI
65K 2353 bp
EcoRV
EcoRI
amp^r

pAbT402
~8520 bp

TK
11K
EcoRI, BglII
BamF
BamHI
lacZ
amp^r
TK
EcoRI
EcoRI

1. NruI + EcoRV
2. BglII linkers
   d(pGGAAGATCTTCC)

1. BglII
2. CIP

FIGURE 4b

Ligate

pAbT6007
~10,880 bp

BglII
11k   65k
BglII
BamF
TK
lacZ
amp^r
TK

Fusion gene and protein generated by ligation of vaccinia 11K
and M. leprae 65kd genes in pAbT6007: nucleotide sequence
at junction of fusion gene and predicted amino acid sequence.

DNA Sequence   ATG   AAT   TCA GAT   CTT   CCC   GAT   CAG   CGA   GTG ----

Predicted Amino
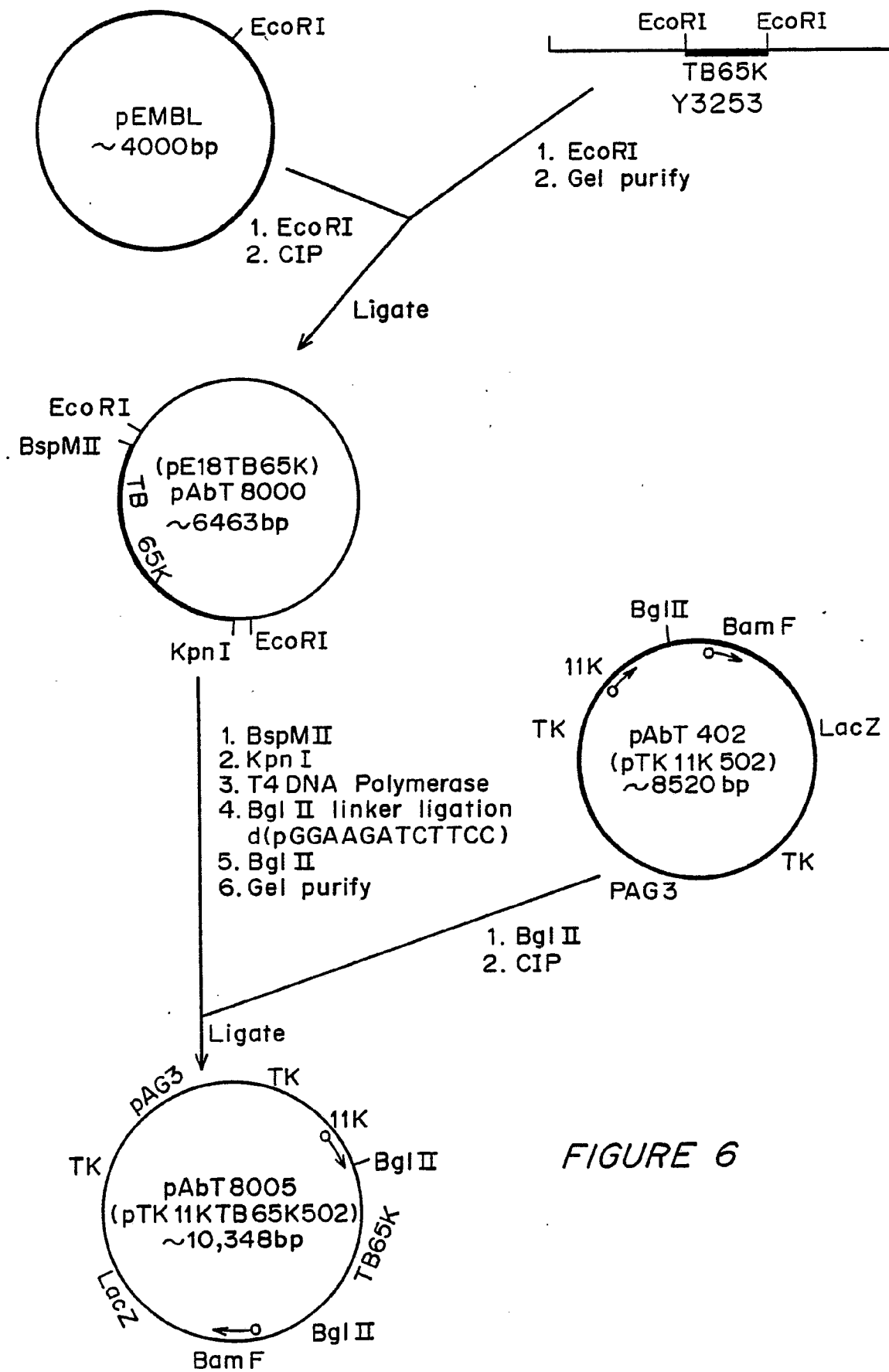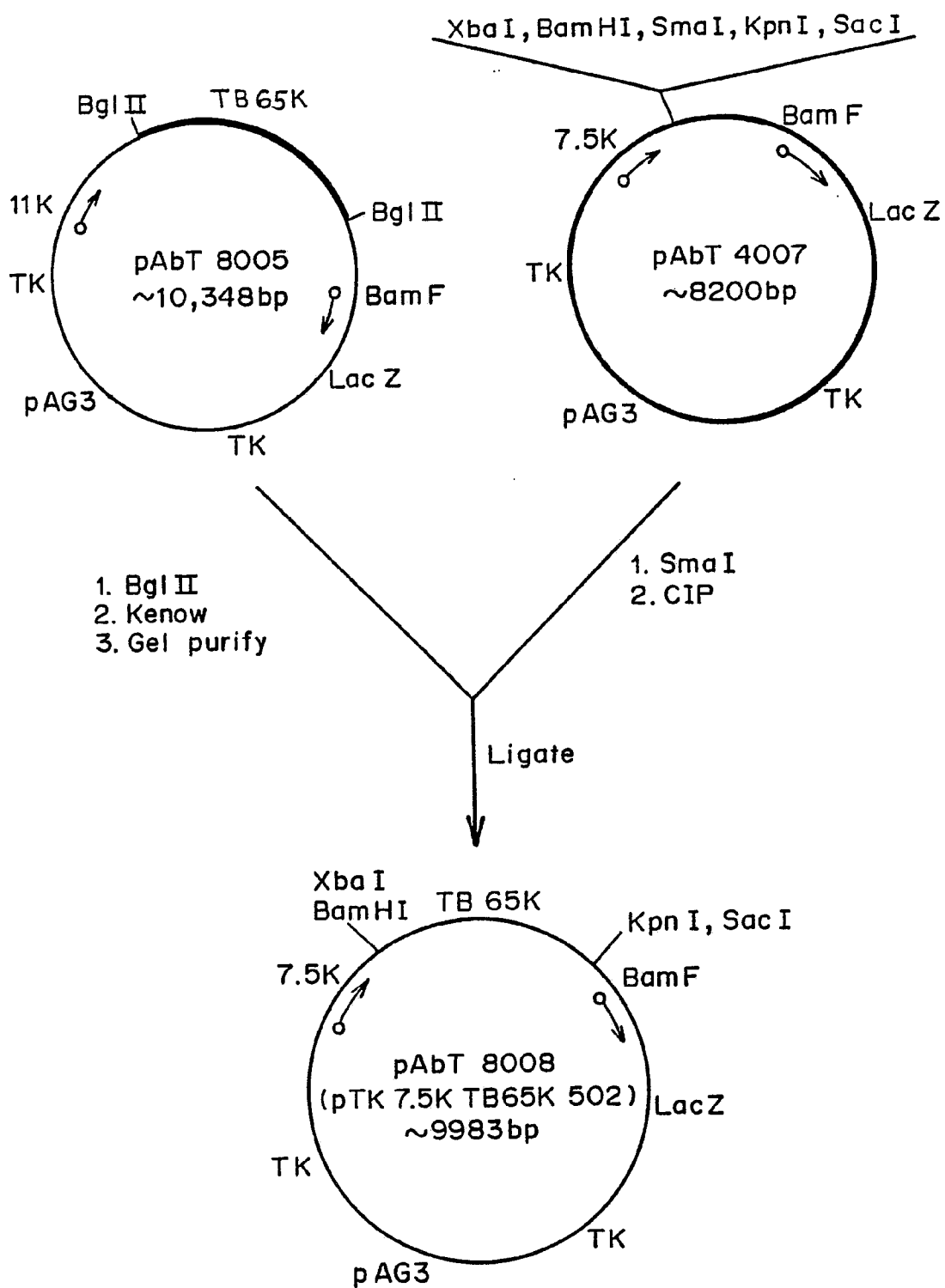Acid Sequence   M   N   S   D   L   P   D   Q   R   V
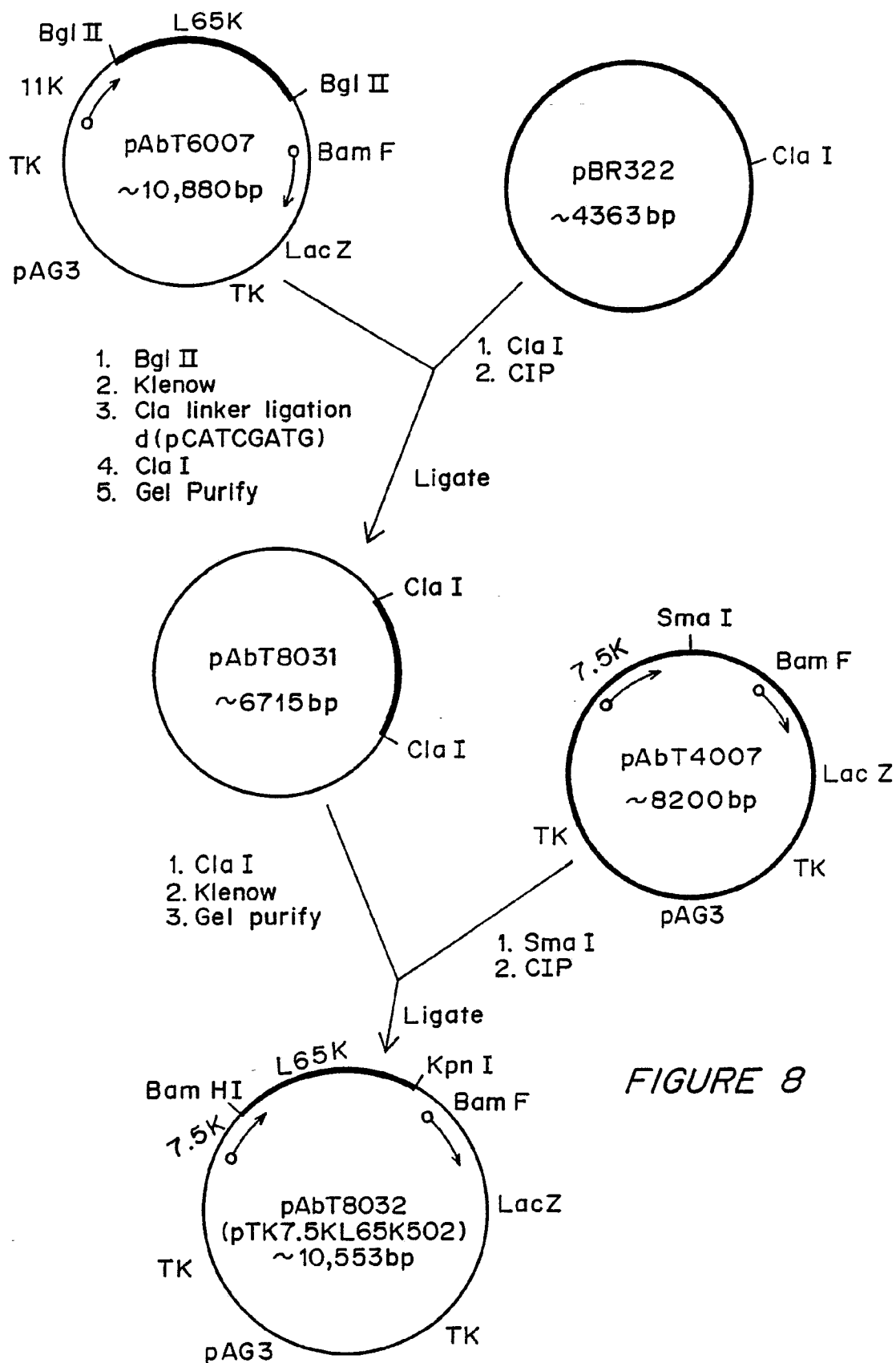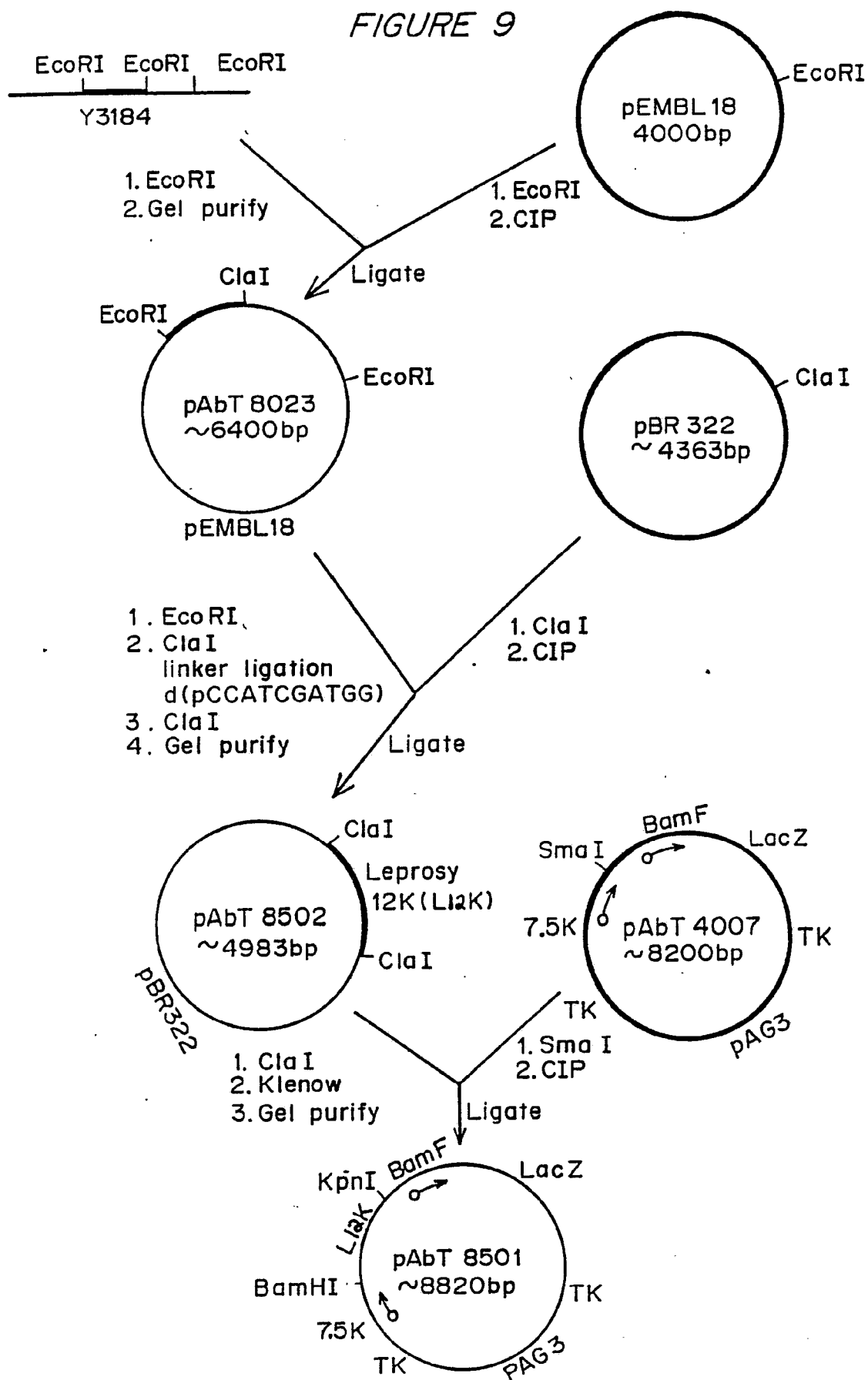
FIGURE 5

FIGURE 6
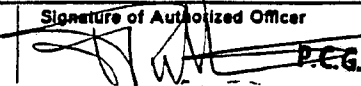
FIGURE 7

FIGURE 8

FIGURE 9

INTERNATIONAL SEARCH REPORT

International Application No   PCT/US 87/02245

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]
According to International Patent Classification (IPC) or to both National Classification and IPC
IPC[4] :   C 12 N 15/00; C 12 N 7/00; // A 61 K 39/04; G 01 N 33/68

II. FIELDS SEARCHED

Minimum Documentation Searched [7]	
Classification System	Classification Symbols
IPC[4]	C 12 N; A 61 K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	Proc. Natl. Acad. Sci. USA, volume 80, September 1983, (US), D. Panicali et al.: "Construction of live vaccines by using genetically engineered poxviruses: Biological activity of recombinant vaccinia virus expressing influenza virus hemagglutinin", pages 5364-5368 see the whole article, especially page 5367, last paragraph cited in the application --	1-15
Y	EP, A, 0110385 (THE UNITED STATES OF AMERICA) 13 June 1984 see claims 10-13 --	1-15
Y	Nature, volume 316, 1 August 1985, R.A. Young et al.: "Genes for the major protein antigens of the leprosy parasite Mycobacterium leprae", pages 350-352 see the whole article cited in the application --	1-5,9-15

* Special categories of cited documents: [10]
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
5th January 1988	- 3 FEB 1988
International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	P.C.G. VAN DER PUTTEN

Form PCT/ISA/210 (second sheet) (January 1985)

Category*	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Proc. Natl. Acad. Sci, USA, volume 82, May 1985, (US), R.A. Young et al.: "Dissection of Mycobacterium tuberculosis antigens using recombinant DNA", pages 2583-2587 see the whole article --	1-3,6,11-14
Y	Infection and Immunity, volume 50, no. 3, December 1985, American Society for Microbiology, (US), J.E.R. Thole et al.: "Cloning of Mycobacterium bovis BCG DNA and expression of antigens in Escherichia coli", pages 800-806 see the whole article	1-3,11-14

III. DOCUMENTS CONSIDERED TO BE RELEVANT   (CONTINUED FROM THE SECOND SHEET)

US 8702245

SA   18735

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 28/01/88
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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
EP-A- 0110385	13-06-84	WO-A-	8402077	07-06-84
		AU-A-	2342484	18-06-84
		JP-T-	60500518	18-04-85

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For more details about this annex : see Official Journal of the European Patent Office, No. 12/82